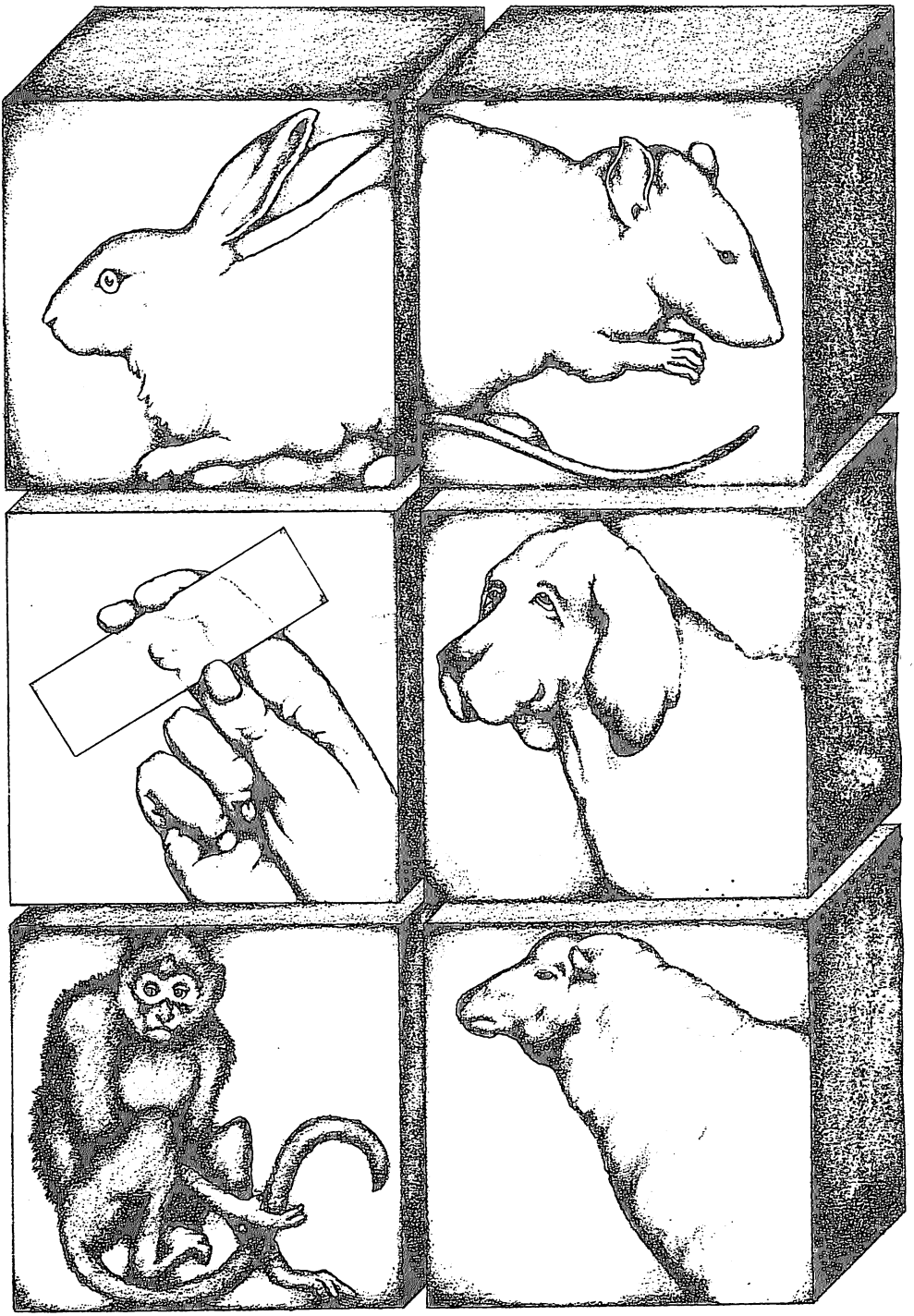
ANIMAL HISTOLOGY PROCEDURES

OF THE PATHOLOGICAL TECHNOLOGY SECTION OF THE NATIONAL CANCER INSTITUTE



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Second Edition

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FOREWORD

Every experiment in cancer research is concerned somewhere in its course with the histological identification and characterization of one or more neoplasms. Often the histological identification is so remotely linked to a long, convoluted chain of experiments concerned with seemingly unrelated phenomena and mechanisms that the connection is at first glance imperceptible. But it has to be there. Experiments in viral genetics, organic chemistry, molecular biology, or immunology have a relatedness to cancer research only if, at some point between the beginning and the end of a chain of experimental procedures, a neoplastic process is involved. Morphological recognition of that neoplastic process is a *sine qua non,* and that recognition is dependent upon technologically suitable preparations whether at light or electron microscopic level.

The prime importance of technological excellence in histologic procedures is, then, self-evident. While we may be approaching the time when immunological and biochemical procedures will exceed structural and biological methods in their ability to resolve differences between normal and neoplastic cell populations, that time is not yet fully upon us. Meanwhile, it will be essential to maintain the highest standards of histological technology, if only because structural interpretations will have to be the meterstick against which advances in immunological and biochemical identifications are measured.

The key role of histopathology in cancer research was no less obvious in 1937 when Dr. Harold L. Stewart set up a laboratory of pathology within the Office of Cancer Investigation, first at the Harvard Medical School in Boston, and later at the Gibbs Laboratory at Harvard University in Cambridge, Massachusetts. A photograph of the Institute staff of those early days can be found on p. 566 of the Journal of the National Cancer Institute, vol. 59, 1977. In the picture is Mrs. Rose Minor, the first histotechnologist of the Office. She is remembered by the few surviving members of the "Old Guard" as a capable worker who turned out first rate slides. Later, Mr. Joseph Stasio, also among those in the early photograph, joined Dr. Stewart's histotechnology staff, and growth for a period thereafter became logarithmic.

In 1939 the Institute moved to Bethesda. By that time the histotechnologic staff consisted of some 5 or 6 workers supervised by Mr. Edgar Nebel. Mr. Nebel left in 1940, to be succeeded by Mr. Albert M. Kessel who had served as Chief Technologist of the Neuropathology Laboratory under Dr. J. H. Globus at Mt. Sinai Hospital in New York City. Mr. Kessel is remembered as a dependable, hard working perfectionist who held a strong disciplinary hand over his subordinates. Among the latter was Margaret Kelly, who subsequently earned a Ph.D. degree, became an investigator in the Laboratory of Biochemical Pharmacology, and, often in collaboration with her pathologist husband, Dr. Roger O'Gara, made many original research contributions. Dr. O'Gara had also come up from the ranks as a technologist with Dr. Stewart's Cambridge staff, to become a physician and then a Board certified pathologist. Mr. Gebhard Gsell, who had worked with Kessel in the Mt. Sinai laboratory, came to Bethesda in 1948 to

become photomicrographer *par excellence,* serving the staff of the Laboratory of Pathology while attached to the Histotechnology Section.

In 1953 an airplane crash in Frankfurt, Germany, took the lives of Albert Kessel, his wife, and their daughter, an only child. Mr. Joseph M. Albrecht, who was at the time a member of Mr. Kessel's staff, replaced Mr. Kessel. Mr. Albrecht had come from Dr. E. V. Cowdry's laboratory in St. Louis. Under Albrecht's innovative influence from 1953 to his retirement in 1969, the Section reached its peak in numbers of technologists and volume of slides produced. Mr. Albrecht's death in Kona, Hawaii in 1972 cut short a happily started retirement.

Ms. Betty Sanders succeeded Mr. Albrecht from 1969 to 1975. Encouraged and supported by Dr. Louis B. Thomas, Dr. Stewart's successor, Ms. Sanders is remembered for her staunchness in maintaining the high standards and productivity of her group in the face of remote administrative pressures that eventually caused reduction of staff to less than half its original strength. She introduced many new procedures, improved the efficiency of the technologists left to her, supervised contract work compensating for loss of staff and wrote the first edition of this manual. Ms. Sanders became one of cancer's victims, but before succumbing she saw to the orderly transfer, without a break in service, of half her staff to quarters in the Frederick Cancer Research Center at Fort Detrick.

From 1975 to the present, the Section on Histopathological Technology has been headed by Mrs. Barbara Coolidge, with Mrs. Ruby Howard in charge of that segment of the section then located at the Frederick Cancer Research Center. Co-authors of this second edition of this manual, their many years of experience through the changing times at NCI have enabled Mrs. Coolidge and Mrs. Howard to recognize and preserve the most useful and reliable of the classical techniques while testing and adding newer methods that must be applied to the modern experimental systems continually being adopted by NCI investigators. During the past decade the Section has metamorphosed from a force of more than 30 workers who performed all the routine and special histological technical service for NCI to a group only a quarter its former size, focusing its efforts on performing special procedures while monitoring the quality of the more routine work done by private contractors. A service in electron microscopy is now offered on a limited basis.

Mrs. Coolidge, Mrs. Howard, and their entire staff have provided a superb service to the investigators of NCI. It is hoped this brief historical review of the evolution of the Section on Histopathological Technology will help place in perspective the important role that Section has played in the drama of cancer research for more than 40 years. Every investigator at NCI is indebted in some way to the competence and industriousness of this group. I welcome this opportunity to express the gratefulness of NCI investigators to the authors and their staff, and to wish them well in their continued striving for excellence.

Clyde J. Dawe

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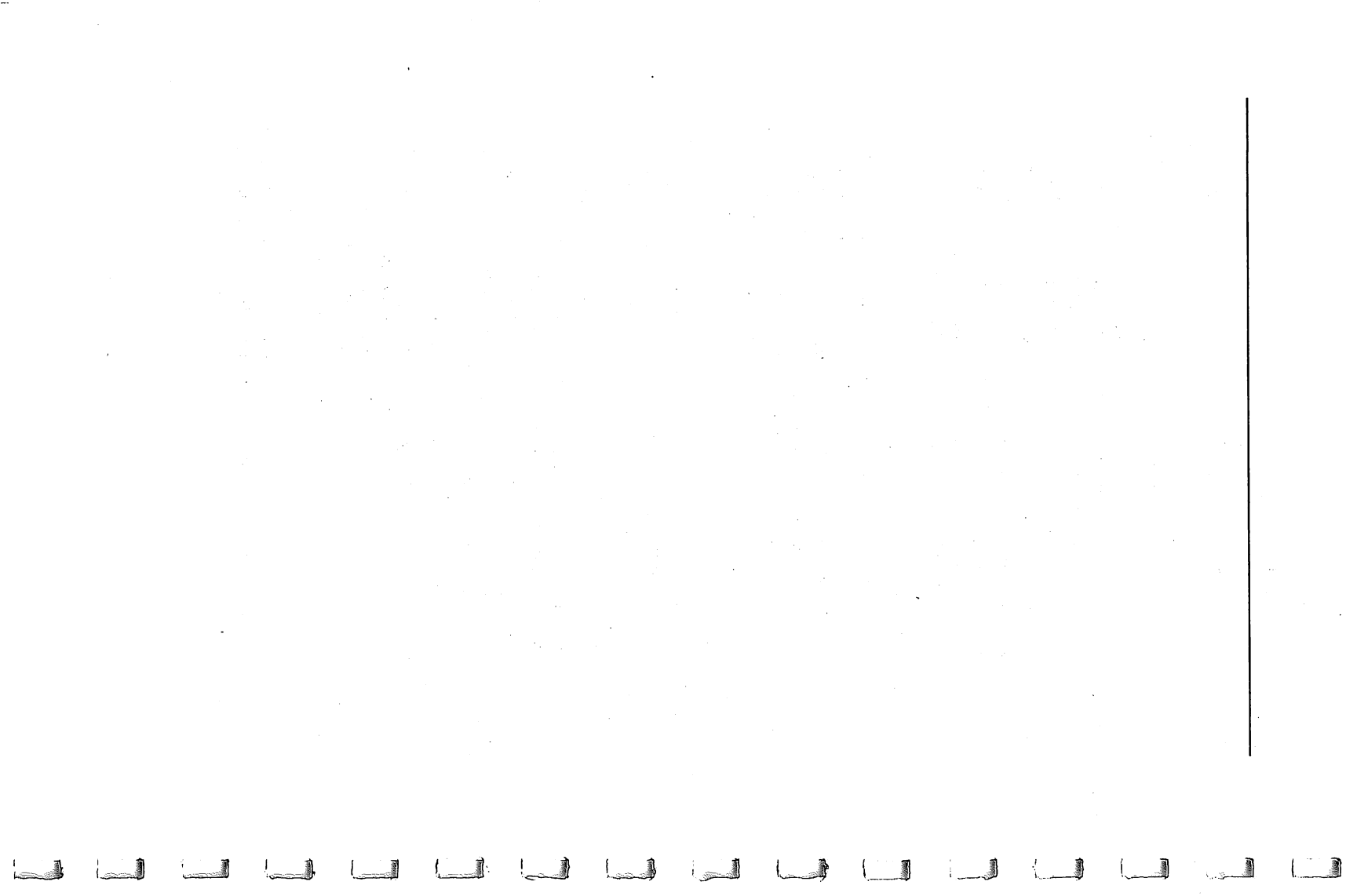
INTRODUCTION

This manual is the reference source used by the staff of the Pathological Technology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The format is similar to the manual, *Histopathology Laboratory Procedures* of the Pathologic Anatomy Branch of the National Cancer Institute, which deals with histopathologic methods for human tissue. The methods selected for laboratory animal tissues in some instances require different or modified histopathologic methods. Animal tissue is submitted to the laboratory of the Pathological Technology Section from approximately 100 investigators of the National Cancer Institute who regularly use mice, guinea pigs, rats, monkeys, dogs, cats, hamsters, swine, reptiles, fish, insects, fowl and invertebrates for their experimental studies. A large volume of the work is for routine histopathology and pathologic screening but in recent years there has been an increasing number of requests for the special techniques and procedures used for specimens in flasks, and on coverslips and whole mounts from investigators who are doing tissue culture, autoradiography, or organ cultures. Many requests are received for thin sectioning of tissue embedded in plastic matrix for light microscopy and ultra thin sectioning for Electron Microscopy. Excellent quality is not only required for the diagnosis of pathologic lesions but is also necessary for photomicrographs for publication.

The stock solution index, pages to is an alphabetic listing of all solutions and their formulae that are kept prepared in the laboratory. An (s) appearing after a solution in the staining procedure refers to that stock solution. Some solutions must be prepared fresh each time used and this is noted. The stock solution index can be helpful in keeping solutions updated and available at all times and provides for an economy of both time and money in a large laboratory. The stock solutions should be carefully labeled. Those requiring refrigeration are labeled with blue tape in our laboratory to distinguish them from the other regular stock solutions. All bottles are labeled with the date of preparation and the anticipated expiration date after which a new stock solution is prepared.

The staining procedures are cross-indexed, pages to , to allow the investigator to select a stain either by name or by tissue component to be stained. Techniques included and described in this manual represent the best methods used for a large number and variety of experimental laboratory specimens and tissues. However, it is anticipated that these techniques will be frequently changed and improved to meet special demands of the future.

Trade names are used for identification only and do not imply endorsement by the Public Health Service or the Department of Health, Education and Welfare.



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SAFETY

There are many hazards in the histology laboratory and safety precautions must be frequently brought to the attention of the technical staff and routinely observed.

Microtome Knives - Full attention must be given whenever knives are being handled to prevent accidents. The hand wheel on the microtome should be locked at all times except when one is actually in the process of cutting.

Glassware -

a. Discard chipped glassware.
b. Use rubber gloves when washing glassware.
c. Wrap containers in several layers of towels when vacuum is used.
d. Use towels or gauze around glass vials or tubing when they are scored and cut.
e. Use safety glasses while etching slides with a diamond pencil.
f. When separatory funnels are used, wrap in towels and loosen top after every turnover.

Explosive and Inflammable Materials - It would be impractical to list all of the explosive and inflammable materials used in the histology laboratory. However, all technicians should be reminded periodically of the dangers and safety precautions which should be observed.

a. Use ether, benzene and all highly volatile materials in a hood. Check adjoining rooms for open flames and warn occupants not to light a flame. Announce to those concerned when danger is over.
b. Most refrigerators are not explosion proof. Do not store volatile solvents in the refrigerator. Use an ice bucket for overnight "cold acetone" solutions.
c. Ammoniacal silver solutions are explosive when dried. Be sure to flush containers and sink well when discarding ammoniacal silver solutions.

Fire -

a. Check all wiring, switches and connections periodically and have electrical equipment properly grounded.
b. Place fire department number on or above every phone.
c. Make sure every employee knows the location of the nearest fire extinguisher and how to use it.
d. Alert every employee on how to secure a room in case of fire.
e. Check gas hoses for leaks frequently.

Chemical Burns -

a. Remind technicians often that chemicals splashed in eyes or on skin must be taken care of immediately.

Probably the most important safety measure is to invite the safety officer to the laboratory to talk to employees about safety procedures and to inspect the facilities regularly. A safety officer often sees potential dangers that are overlooked by those not trained in safety precautions.

METHOD TO RECOVER MERCURY FROM USED FIXING FLUIDS CONTAINING MERCURIC CHLORIDE

Mercury pollution of sewage and streams is a problem and a pathology laboratory can contribute to the problem unless efforts are made to remove mercury from fixatives before they are discarded.

The procedure which is used in our laboratory was reported in the Technicon Bulletin, by Dominic L. Europa, Supervising Histo-Cytologist in the Pathology Laboratories, Bellevue Medical Center, New York, N.Y.

1. Collect fixatives which contain mercury in a jug. Store in a hood or well ventilated area.
2. Add 20 ml. of 13% thioacetamide to each liter of mercuric chloride waste. The 13% thioacetamide can be stored in a capped glass container and is stable for approximately one year.
3. Mix thoroughly and allow the solution to stand in a hood for 24 hours. The solution can be capped.
4. After 24 hours, filter the solution in a hood with fluted filter paper. The clear filtrate can then be discarded. The residue containing mercuric salt can be stored in a glass jug indefinitely.

ACID CLEAN GLASSWARE

Dichromate-Acid Cleaning Solution

Potassium dichromate ..... 200 gms.
Distilled water ..... 2000 ml.
Dissolve with low heat.
Add *slowly* -
Sulfuric acid ..... 200 ml.

Extreme care must be taken in preparation of this solution and its use. Take the following precautions.

1. Add the acid to the aqueous solution - slowly. Place container in stone sink while pouring acid.
2. Wear protective face mask, acid resistant apron and gloves. Check gloves for holes each time they are used.
3. Potassium dichromate has allergenic properties and should be handled with care.
4. Store in acid proof container.

Clean glassware is submerged in the cleaning solution for two hours or more, rinsed well in running water and several changes of distilled water. Dry and store in a dust free cabinet.

To dispose of old acid bath, use safety waste containers.

DO NOT POUR DOWN THE DRAIN.

CELLOIDIN - for Coating Slides

This procedure is used when tissue tends to come loose from the slides.

Prepare:

0.5% Celloidin Solution

1% CELLOIDIN (s) ..... 20 ml.
Ether, anhydrous ..... 20 ml.

Procedure:

1. Xylene - 2 changes.
2. Absolute alcohol - 2 changes.
3. Place in 0.5% Celloidin for 1 minute.
4. Allow sections to dry until white.
5. Place in 70% alcohol for 3 minutes.
6. Wash in running water.
7. Proceed with stain.

If Mercuric Chloride fixative is used -
Drain celloidin from slide and place in Iodine for 3 minutes, rinse in 70% alcohol and place in Hypo for 3 minutes. Wash in running tap water. Stain as desired.

Most of the celloidin will be removed by the absolute alcohol in dehydration. The dehydration and clearing steps should be done rapidly and mounted as soon as possible. Celloidin solution is highly explosive and should be stored and used in a hood.

EGG ALBUMIN

1. Break eggs and separate yolks* from whites. (From one egg to two dozen.)
2. Be sure no yolk is allowed to get into the whites.
3. Break into porcelain dish. (CLEAN AND DRY)
4. Cut the strands of the egg white as fine as possible with scissors.
5. Do not stir egg whites, as this will cause bubbles.
6. Filter egg whites. Use three thicknesses of gauze.
7. Measure the cut egg whites and add an equal amount of glycerin. (FRESH-UNOPENED).
8. Add a few crystals of Thymol.
9. Date bottle.

Egg albumin MUST be kept in the refrigerator. Each technician puts 5-8 ml. in a small plastic squeeze bottle and stores it in the refrigerator when it is not in use. The albumin and bottle are discarded after approximately 30 days and a fresh supply from the stock bottle is put in a new plastic bottle. The albumin is a good culture medium and can easily become contaminated. Bacteria in the albumin can appear to be in the tissue. Use egg albumin sparingly on alcohol cleaned slides.

*Save yolks for a "Sunshine Cake."

PLASTIC SECTIONS FOR LIGHT MICROSCOPY

Pieces of tissue with maximum dimensions of 10 mm x 5 mm x 2 mm can be processed, embedded in methacrylate and cut as thin as 0.5 microns. The tissue is not exposed to prolonged heat and xylene as in the usual paraffin technique which causes shrinkage and distortion of cells. Resulting sections are essentially free of artifacts. The ability to obtain 1-3 micron sections for autoradiographs, needle biopsies, bone marrow biopsies etc. has many obvious advantages. In addition to hematoxylin and 1% Phloxine B, sections can be stained by many of the histochemical and histological staining procedures used on paraffin sections.

Blocks are cut on a JB-4 Sorvall microtome using a glass knife. Other microtomes may be used by purchasing a glass knife holder for rotary microtomes.

Supplies needed:

1. JB-4 Sorvall microtome
2. JB-4 Plastic embedding kit* - this consists of:

Solution A - Glycol methacrylate, butoxyethanol and benzoyl peroxide.
Solution B - Polyethylene glycol 400 and N, n-dimethylaniline.

3. Plastic block holders*
4. Omni-cup molds*

Fixation:
Any

Dehydration:
Dehydrate well in graded alcohols or acetone. Maximum dimensions of the tissue must not exceed 10 mm x 5 mm x 2 mm.

Infiltration:
Infiltrate in several changes of Solution A for 1-15 hours depending on specimen size. Vacuum hastens process and is helpful with dense tissues.

Embedding:

1. Mix thoroughly 42 parts Solution A with 1 part Solution B. This mixture should be prepared just prior to use as it will start to polymerize in less than an hour.
2. Pour 2 ml. of the embedding mixture in the Omni-cup mold.
3. Place specimen in the mold in the proper orientation and cover with plastic block holder.
4. Pour hot paraffin around holder to seal.
5. Allow mold to polymerize for 3-7 hours at 20-30°C.
6. Remove holder from mold. Non-polymerized media may be removed with absolute alcohol.

The specimen is ready for sectioning.

Sectioning:

1. Trim excess plastic from face of block with a glass knife and insert a new glass knife.

2. Turn the microtome handle slowly and as the section comes off the glass knife it is pulled down along the knife with pointed forceps.
3. The section is held above a vessel of room temperature water and gently blown to the surface of the water.
4. The section is picked up on alcohol cleaned slides. No adhesive is necessary.
5. Dry sections in over at 60°C. overnight.

Staining:

Hematoxylin and 1% Phloxine B.

The plastic matrix is not removed.

1. Mayer's hematoxylin for 30 minutes.
2. Blue in several changes of tepid tap water.
3. Rinse in distilled water.
4. Stain in 1% Phloxine B (s) for - 2 minutes.
5. Wash in running water - 5 minutes.
6. Dry in 60° oven - 15-30 minutes.
7. Xylene
8. Mount

*Polyscience, Inc., Paul Valley Industrial Park, Warrington, Pa., 18976.

INSECT PROCESSING SCHEDULE

Fixative — Bilstad (other fixatives may be used).

Soak tissue in phenol solution for 24 hours after fixation.

Prepare:

Phenol Solution

Phenol (melted) ..... 4 ml.

80% Alcohol .....96 ml.

Step	Water	Ethyl Alcohol	n-Butyl Alcohol	Phenol	Time
1	95 ml.	5 ml.			1/2 hr.
2	90 ml.	10 ml.			1/2 hr.
3	80 ml.	20 ml.			1/2 hr.
4	65 ml.	35 ml.			1 hr.
5	50 ml.	40 ml.	10 ml.		1 hr.
6	30 ml.	50 ml.	20 ml.		1 hr.
7	15 ml.	50 ml.	35 ml.	4 ml.	24 hr.
8	5 ml.	40 ml.	55 ml.		1 hr.
9		25 ml.	75 ml.		1 hr.
10			100 ml.	4 ml.	1 hr.
11			100 ml.	4 ml.	1 hr.

Infiltrate with 3 changes of paraplast, 3 hours each change and embed. Trim and cut excess paraplast from face of block and soak overnight in tap water. Cut, float ribbon on 35% ethyl alcohol, flatten on 46° warming tray, drain and dry.

Reference: Smith, Stanley G., Stain Technology, Vol. 15, No. 4, Oct. 1940

WORM PROCESSING SCHEDULE

After fixation place in - Butyl alcohol - 2 changes - 6 hours each.
Butyl alcohol saturated with paraplast - 24 hours in 60° oven.
Paraplast - 24 hours.
Embed in paraplast.

SPECIAL TECHNIQUES

1. Serial Sections
We recommend the use of a "soft" fixative such as 10% Formalin or Bouin's to eliminate the necessity of soaking blocks which are to be sectioned serially. If "hard" fixatives such as Zenker's fluid are used the block can be soaked on the microtome with gauze wet with 5% detergent. The ends of the gauze can be wrapped around the adjusting screws of the microtome to hold it snugly against the exposed tissue. Because this is time-consuming the technician often uses three microtomes, each with a block to be serial-sectioned. Two blocks can then be soaking while a ribbon is cut from the third block.

Paraffin blocks for serial sectioning are not rough trimmed and are not removed from the microtome once sectioning has started. Complete serial sectioning means that the first ribbon of the paraffin sections includes the first cell of the tissue and the last ribbon contains the last cell of the tissue in the paraffin block. As many ribbons as will fit under a 50 mm. coverglass are placed on each slide so that the serials are in order and each glass slide is consecutively numbered. Serial sections are placed on a 46-50° warming plate to eliminate wrinkles in the sections. It is difficult to float serial sections on a water bath and pick them up on glass slides without either losing sections or getting the serial sections out of order.

Step-Sections (at prescribed levels)
When it is not necessary to cut complete serial sections but the investigator wishes to examine sections from several levels in the block, step-sections are cut. Examples of requests for step-sections are:

a. Cut at 3 levels
b. cut at 10 levels
c. alternately save 3 sections and discard 30 sections throughout the block.

2. Millipore Filters
Cells on millipore filters are usually stained with H & E but are sometimes stained with the 8 minute Giemsa method or with other staining procedures. Absolute methanol dissolves the filters but they may be fixed in 80% methanol. Other suggested fixatives are Telly's, Lillie FAA, Gluteraldehyde or 10% Formalin. The acid fixatives produce sharper staining. If more than one stain is needed the filters may be cut in half or in quarters. Millipore filters which are stained before being mounted on slides require more time in xylols to clear before coverslipping. Excess permount is needed under and on top of the filter so air bubbles will not be trapped under the coverglass. Millipore filters may also be embedded in paraffin and sectioned.

3. Coverglasses and Flasks used in Tissue Culture
These require longer staining times usually because of the alkalinity of the culture fluid. Care must be taken with plastic flasks because xylol will dissolve some of them. After differentiation they can be air dried and

covered with a thin layer of immersion oil or mineral oil to get good high power resolution with the microscope. Many types of viral inclusion bodies can be demonstrated with an H & E stain. Lillie FAA is a good fixative, but other types of fixatives may be used.

4. Segregated or dispersed cells in fluids

Many preparations submitted to the laboratory have dispersed, free-floating cells in various fluids. These may be bone marrow cells, buffy coat preparations or pieces of tissue. They are usually processed by being centrifuged after each step in fixation, washing, dehydration, etc. The fluids should not be decanted but should be drawn off with a capillary pipette using vacuum and a side arm flask as a trap. The cells are then mixed with the next solution. After being cleared in xylol and centrifuged, the xylol is removed and paraplast is carefully added to the button of cells at 60°C. The paraplast is changed several times with a hot capillary pipette using a rubber bulb for suction. When thoroughly infiltrated the centrifuge tube is taken from the oven and allowed to harden. The tube can then be broken or the outer surface of the paraplast can be gently melted with a flame and the block of paraplast slipped out of the tube and embedded in a receptacle containing additional paraplast. Over-heating during this step may cause the entire block of paraplast to melt and the button of cells or tissue fragments may be lost.

5. Extremely Small Specimens

Examples of extremely small specimens are mouse embryo organs such as the thymus or an intact hydra. These specimens often measure less than a millimeter in diameter but it is necessary that they be processed and sometimes it is also requested that they be oriented in a particular plane before being sectioned. The first step is to make the specimen more visible by coloring it with 0.5% ALCOHOLIC EOSIN (s) for a short time. Then the specimen is handled in the same fashion as described for free floating cells except that they are placed in absolute alcohol immediately because 95% alcohol would remove the eosin. The specimens are embedded in small stender dishes with the aid of the dissecting microscope to assure proper orientation. The stender dishes are first coated with a thin coat of glycerin. This facilitates the removal of the hardened block. Disposable capillary pipettes with rubber bulbs are used to transfer the specimens to the embedding dish and to manipulate the specimen into the correct position. The pipette tips are warmed repeatedly with a flame.

6. Slides for Radioautography

Routinely sections are cut at 4 microns and mounted on GELATINIZED SLIDES (s). The sections are deparaffinized and brought to water. They are returned to the investigator to be covered with emulsion. They are then returned to the laboratory for staining, usually with H & E. Many investigators request only light staining so that silver grains in the emulsion can be easily seen.

FROZEN SECTIONS

Frozen sections are cut when there is some component in the tissue which will be destroyed by heat or dissolved in the solutions used for routine tissue processing.
The tissue may be fresh and unfixed or it may be fixed in 10% Formalin. If in Formalin, it is washed in water, frozen on the freezing microtome and cut at 10 microns. Sections may be picked up on albuminized slides or gelatinized slides. Sections may also be carried through the stains singly on a glass or metal rod and placed on clean slides for mounting. Those stained for fat must be mounted in glycerin - jelly as alcohol dissolves many lipids.

Gelatin Embedding

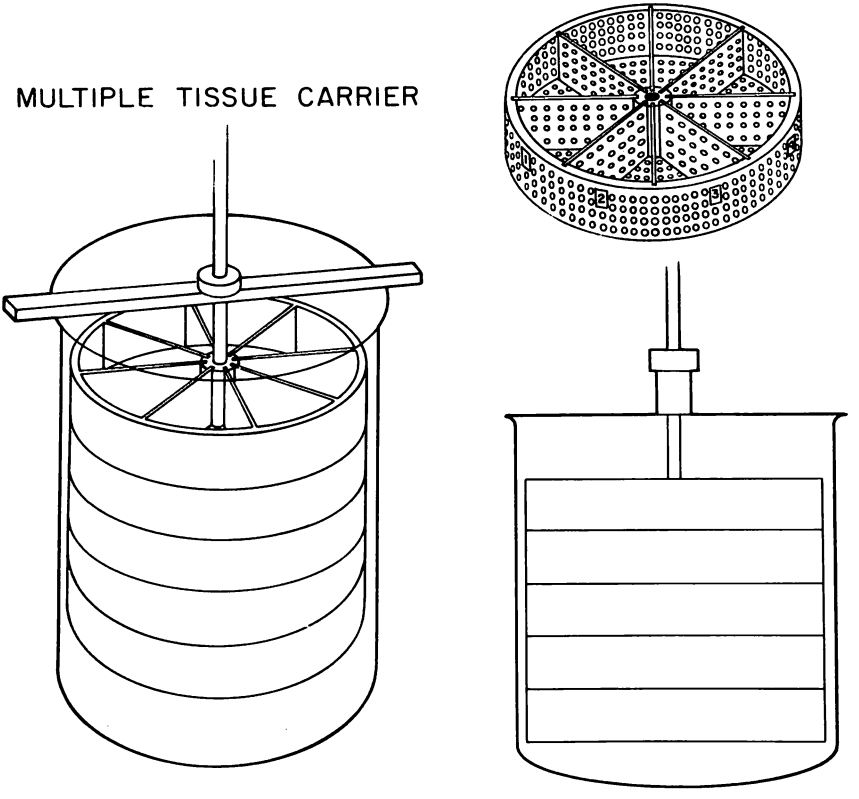
1. Wash tissue in water.
2. Infiltrate with 6% gelatin in 37°C. vacuum oven for one hour.
3. Infiltrate with 12% gelatin in 37°C. vacuum oven for one hour.
4. Embed in 12% gelatin and place in refrigerator until firm.
5. Cut excess gelatin from the block with a sharp razor blade.
6. Place block in cold 10% formalin in the refrigerator overnight.
7. Wash the hardened block in water and cut sections on the freezing microtome. Stain sections with the same methods as any fixed frozen tissue.

Gelatin embedding is used for tissue that is fragmented and for small pieces of tissue.

Cryostat Sections

Unfixed tissue is sectioned on the Cryostat when there is a component that may be destroyed or altered by fixation and processing and when immediate examination of fresh tissue is desired.
Fixation, which is necessary when tissues are cut on the freezing microtome, provides multiple barriers to many procedures involving enzyme histochemistry, fluorescence microscopy, protein and inorganic histochemistry.
Cryostat cut tissue sections which are mounted and fixed correctly eliminate all the artifacts found in paraffin sections which have undergone severe chemical, solvent and heat changes.

Figure 1—*Multiple tissue carriers*



The multiple tissue carrier invented by Mr. Albert M. Kessel* has five trays each of which has eight perforated, removable compartments. The compartments are numbered from one to forty. The carriers have many advantages for processing large numbers of tissues. They decrease the possibility of error because it is simple to load them with tissue and tissue can be directly removed from the carrier compartments for embedding. The compartments can be removed to accomodate tissue of various sizes and to facilitate cleaning. As the carrier is loaded the accession number and compartment number are written on an embedding sheet. As the tissues are embedded the compartment number of the embedding tray is added to the sheet. The embedding sheet is kept as a permanent record and is often helpful as a double check on the information in the technician's work book.

*Patent No. 2,302,698. Mr. Kessel was Head of the Pathological Technology Section from 1937-1953.

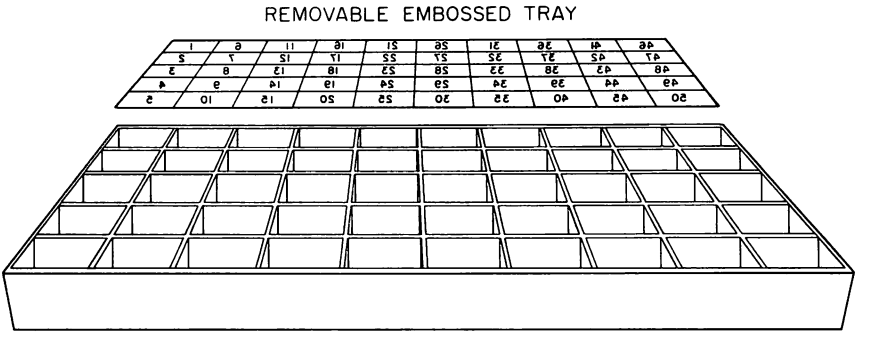
ROUTINE TISSUE PROCESS SCHEDULES WITH VACUUM TISSUE PROCESSOR

	5mm +	up to 5mm	up to 4mm	up to 3mm	up to 2mm	up to 1mm
	16 hr.	8 hr.	6 hr.	4 hr.	2 hr.	1 hr.
80% Ethanol	30	20	20	10	6	5
95%	60	20	20	10	6	5
95%	30	30	30	10	6	5
100%	60	40	30	20	6	5
100%	180	40	30	20	6	5
100%	180	40	40	20	10	5
100%	180	60	40	30	10	5
Xylol	30	20	20	20	10	5
Xylol	30	30	30	20	10	5
Xylol-Paraplast	60	60	30	20	10	5
Paraplast	60	60	30	30	20	5
Paraplast	60	60	40	30	20	5
Paraplast*	30	20	20	20	20	10

*A portable vacuum paraffin bath is used after tissues are removed from the tissue processor. This is necessary for complete infiltration. Steps 1,6,7,8,9,11,12 changed after every use. Other solutions are changed frequently.

Embedding

Figure 2-*Embedding Tray**



Embedding trays of various sizes and with variable numbers of compartments and with removable, numbered plates are excellent for embedding large numbers of tissues. The largest tray used in our laboratory is 24" x 7.5" x 1 1/2" and has 50 compartments. A removable plate which fits under the compartments is embossed with numbers so that each paraffin block is automatically numbered. The embedding trays are placed on a large hot plate which is kept at 65°C. until embedding is completed and then the paraplast is cooled, first in running water and then in the refrigerator. The tray can be disassembled to remove the paraffin blocks. The embedding tray shown in figure 2 has 50 compartments and there are smaller trays with 25 compartments.

Excess paraplast is recycled into the infiltrating baths.

Compatible tissues should be embedded together such as liver and lung, not skin and brain. All tissues should be placed as close as possible to each other without touching, flat, horizontal, and on the same plane. Small organs such as mouse pituitary or thyroid should be embedded alone or with tissues of similar size. Eyes must be embedded separately for good orientation of the optic nerve.

If tissues are embedded poorly, some organs will be cut away while attempting to get complete sections of others and representative samples are not obtained.

Tissue carriers of the compartmented tray type are preferable to cassettes because all tissues of one animal are available together and the technician can be selective as to which tissues to embed together.

*Kessel, A., Multiple Tray for Embedding Tissues in Paraffin.; J. Nat. Cancer Inst., 257-9, 1945.

DOUBLE EMBEDDING

The double embedding technique is used on tissues which are difficult to section when good quality is essential.

Paraffin Embedded Sections

1. Melt paraffin from tissue in oven.
2. Clear in toluene 1-2 hours (2-3 changes.)
3. Absolute alcohol -3 changes. 30 minutes to 1 hour.
4. Place in 1% CELLOIDIN IN METHYL BENZOATE (s) to which has been added an equal amount of alcohol. Place tissue gently on top of the solution. Tissue will gradually sink into celloidin. (12-24 hours)
5. Transfer tissue to 3% METHYL BENZOATE CELLOIDIN (s) for 48-96 hours.
6. Place tissue directly into benzol and leave overnight.
7. Infiltrate in 58-60°C. oven in paraplast - 3 or 4 changes. Leave in oven overnight. (If vacuum oven is used - 1/2 hour each change.)
8. Embed in paraplast and cut.

Steps 4, 5 and 6 should be carried out in a hood.
For tissue received in 70% alcohol - dehydrate to absolute alcohol and continue from number 3.

Reference: *Laboratory Technique,* Cowdry, 3rd. Edition, p. 103.

SECTIONING PARAFFIN BLOCKS

The following steps are taken to process and section paraffin blocks:

1. Accession numbers for each paraffin block are entered in the Technician's Work Book.
2. The paraffin block is trimmed and labeled with a typewritten accession number.
3. The block is rough-trimmed with a sharp microtome knife to expose the full surface of the tissues to be sectioned.
4. Paraffin blocks are soaked in water for variable lengths of time depending on the tissue and the fixative used. Formalin and Bouin's fixed tissues require less soaking than tissues from other fixatives. Zenker fixed tissues sometimes require soaking overnight or longer. Tap water is usually used. 5% detergent water may be used for faster soaking but the blocks must be carefully watched and never left overnight. If left too long the tissues will swell. 50% Glycerin-water is used to soak paraffin blocks containing bone or cartilage. Many tissues of laboratory animals are more difficult to section than human tissue because they seem to be more brittle and float off the slide more easily during the staining process. Soaking helps prevent this. Paraffin blocks used for cutting serial sections can be soaked on the microtome with gauze saturated with 5% detergent water.
5. Paraffin blocks are iced with an ice cube.
6. Sections for H & E staining are usually cut at 5-6 microns and are mounted on albuminized slides. All slides and coverslips are alcohol cleaned before use and marked with the appropriate accession number. Wrinkles are removed either by floating the sections on a warm water bath or by placing the glass slides on a warming tray. If the paraffin sections are cutting well and are large, the water bath is best for removing wrinkles. If the sectioned tissue is small or difficult to cut, the warming tray is better. The warming tray *must* be used to cut serial sections and step-serial sections.

A sharp microtome knife is an absolute necessity for good sectioning. There are many good knife sharpeners available and several are used in our laboratory with good results. A properly sharpened blade will cut 3 micron sections with no compression. Stropping is unnecessary if the knife is properly sharpened, but a well stropped knife holds the cutting edge longer.

7. After sections have been cut, the paraffin blocks are filed in No. 2 pill boxes and kept in cabinets with large map-file type drawers. Proper identification and easy access to the stored blocks are important because in our laboratory the blocks are frequently recut for special stains and for additional slides for study sets, workshops, or other investigators.

TRANSFER OF TISSUE FROM BROKEN SLIDES

When it is not desirable to mount a broken slide on another slide because of excessive thickness (when high resolution is needed on the microscope) or if a slide has been broken in too many pieces, we use the following method.

1. In a shallow pan assemble all pieces of broken slide on another slide like a puzzle.
2. Slowly pour xylene in pan until slides are covered.
3. Soak until all pieces of coverslip can be easily removed. Lift slides from container and place on paper towel. While broken pieces are still wet with xylene pour Diatex* (plastic) on entire slide..
4. Dry overnight at room temperature or in a 60° oven for 2 hours (plastic will be hard).
5. Cut around edge of slide with a razor blade.
6. Soak in tap water (about 2 hours) until plastic can be easily peeled from slide.
7. Trim around tissue with scissors.
8. Place plastic coated section on clean albuminized slide. Cut a paper towel the size of the slide, dampen it, and place it on top of the plastic film. Place a clean slide on top. An object should be placed on top of slide to hold it in place. Leave at room temperature or place in 37° oven overnight (towel will be dry).
9. Remove top slide. Put slide with film and paper towel into xylene. The towel will soak loose, and the slide should remain in xylene until the plastic is completely dissolved.
10. Coverslip.

*Scientific Products, Inc.

ROUTINE STAINING

Hematoxylin and Eosin is the routine stain used in our laboratory and the schedule is as follows:

1. Xylene - 2 changes ..... 2-3 minutes each
2. Absolute alcohol - 2 changes ..... 2-3 minutes each
3. 95% alcohol - 1 change ..... 2-3 minutes
4. IODINE SOLUTION 0.5% (s) ..... 3 minutes
5. 70% alcohol ..... several dips
6. HYPO (s) ..... 3 minutes

(steps 4, 5 and 6 are necessary only when tissues are fixed in a mercuric chloride solution.)

7. Wash in running tap water.
8. Rinse well in distilled water.
9. MAYER'S HEMATOXYLIN (s) ..... 30 seconds - 2 minutes
10. Rinse in running tap water to remove excess stain and place in lukewarm water to "blue" sections. (If slides stay in water for a long period we have found that the chlorine will bleach the hematoxylin.)
11. Rinse in distilled water.
12. 50% alcohol ..... 1-2 minutes
13. 70% alcohol ..... 1-2 minutes
14. 0.5% ALCOHOLIC EOSIN (s) slightly acidified with acetic acid 5 dips - 30 seconds
15. 95% alcohol - 3 changes ..... 30 seconds each

During these steps the slides are agitated to remove excess eosin. If this is not done the overlay of the eosin gives the sections a muddy appearance. The 95% alcohol is changed frequently.

16. Absolute alcohol - 2 changes ..... 1-2 minutes each
17. Alcohol - xylene ..... agitate - 30 seconds

If sections look milky, dehydration is not complete. Take back to clean absolute alcohol and return to fresh alcohol-xylene.

18. Xylene - several changes until sections are clear.
19. Mount

The stained sections are covered with permanent mounting medium and coverglasses which have been cleaned in alcohol. Clean gauze and xylene are used to clean excess resin from the slides and from the margins of the coverslips. The slides are labeled with the accession number, fixative, investigator's name and are initialed by the technician who cut, stained and mounted the sections. They are then checked out of the Technician's Work Book, inspected for quality by the supervisor and boxed for the investigator.

All times noted in the stain procedure can be decreased if the slides are agitated. Acid-fixed tissues require less staining time for both hematoxylin and eosin. Formalin-fixed tissues require the maximum times. Reptile, fowl, embryo and tissue culture cells usually require maximum staining times especially when fixed in 10% formalin. If too much eosin washes out in the 95% alcohol, and the result is a pale contrasting stain, add 1-2 drops of glacial acetic acid to the 95% alcohols after the eosin.

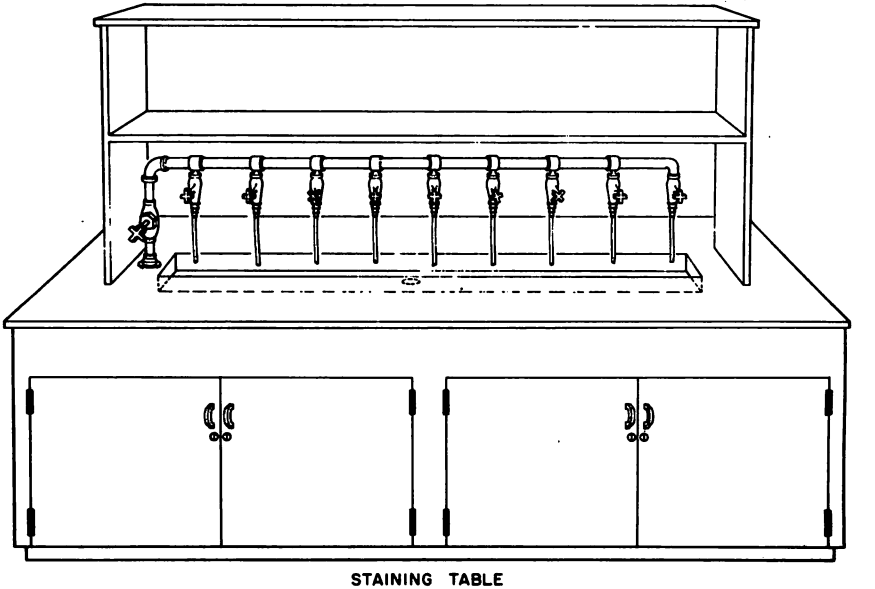
Note No. 1.
Here as elsewhere in the manual when alcohol is mentioned we are referring to ethyl alcohol unless otherwise specified. Also when water is mentioned we mean distilled water unless tap water is specified.

Note No. 2.
In the Staining Procedures portion of this manual the first step usually is "Deparaffinize slides and hydrate to water." This always means taking sections through Iodine, 70% alcohol and Hypo (as in steps 4, 5, and 6 of the routine H & E) if the fixative contains mercuric chloride. This process removes the mercuric chloride crystals.

In the staining and mounting procedures two important pieces of equipment are used. Both were designed by Mr. J. M. Albrecht, Head of the Pathological Technology Section from 1954 to 1968. One is a staining tabel (figure 3) which is placed in the middle of the laboratory room and the other is a plastic, portable hood (figure 4) which is placed on the laboratory bench and is used for cover slipping.

The staining table placed in the middle of the room is a space saver because the technicians can walk around it and use it from both sides. The tables used in our laboratory are 96" x 30" and large staining dishes can be placed on both sides of the table top. The stainless steel trough in the center is 60" long and 8-1/4" wide and is used for washing slides. Shallow pans are kept in the central trough to hold racks of slides which are being washed. The cabinets in the staining tables provide convenient and easily accessible storage for the solutions used in the staining procedures.

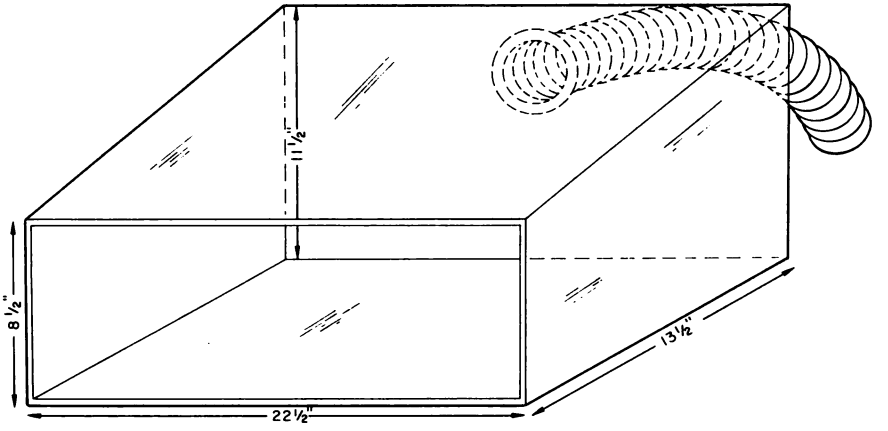
Figure 3 - *Staining Table*



STAINING TABLE

The plexiglass portable hood with an exhaust attachment is used for cover slipping and is the most important innovation in this laboratory in recent years. It is comfortable to use, provides good visibility and reduces to a minimum inhalation of toxic xylene fumes. The hood is used when manipulating any unpleasant or dangerous volatile chemical. The flexible exhaust hose which is reinforced with wire connects to the air exhaust system. It can be detached with ease and the hood can be removed or stored when not in use.

Figure 4 - *Hood for Mounting Cover Slips*



PORTABLE HOOD

METHOD OF RECORD KEEPING

A permanent, bound accession book should be maintained so that all samples and tissues submitted to the laboratory can be given accession numbers and these accession numbers should be used to identify all blocks and slides which are prepared in the laboratory. Pre-numbered accession books can be designed using appropriate combinations of letters and numbers. The accession books used in the Pathological Technology Section are obtained from the Government Printing Office NIH-644 (Formerly PHS-815) Rev. 3-65. Each entry for a tissue or a specimen in the accession book should include the investigator's name, date of accessioning, number or identifying symbol for his experiment, etc.

In addition to the accession book, we have found it useful to have permanent work books for the technicians. We use Accession Record Book NIH-695 (8 1/2 x 11 inches). Information in these includes Accession number, Investigator's name, Technician's Initials, Fixative, Number of Pieces, Number of Blocks, Number of Slides Routine (hematoxylin and eosin stained), Number of Slides Unstained, Date Blocks were Received, Date Sections were Returned, Special Stains, and a column for Other Remarks. This book provides an excellent reference on all work that has been performed in the laboratory.

At the time each investigator first submits tissues or materials for processing in the laboratory he is given an information sheet for fixing and submitting tissues.

PROCEDURE FOR SUBMITTING TISSUE TO THE PATHOLOGICAL TECHNOLOGY SECTION FOR PROCESSING

1. New investigators fill out form indicating Building number, Room number and Laboratory or Branch to which connected.

2. Fixation and Trimming of Tissues

(a) Tissue, to be properly fixed, should not be over 5-6 mm. thick.

(b) Fixative used should be one which will give best results for desired staining procedure.

(c) Trim tissue so that the area from which sections are to be taken will be on or near surface of block.

(d) Tissues fixed in Zenker's fluid or other fixatives containing corrosive sublimate should be washed in running tap water for at least 12 hours. Tissues fixed in Bouins should have 3 changes of 70% alcohol. Tissues fixed in Formalin or like solutions should have excess formalin washed off before being placed in 70% alcohol.

(e) All tissues containing bone or calcific deposits must be thoroughly decalcified and washed for 24 hours in running tap water before being submitted.

(f) Excess hair should be clipped from skin.

(g) It is advisable to place all thin membranes or skin on pieces of index card before fixation so as to facilitate the cutting of transverse sections.

(h) All tissues will be in 70% ethyl alcohol when submitted unless otherwise indicated because of a special technique.

(i) One slide stained with Hematoxylin and Eosin in the routine procedure.

3. Information required when submitting tissue.

(a) The following information is to be placed on WHITE tag on bottle (white tag available through NIH stock Catalog No. 5-8666).

1. Investigators Name-2. Fixative-3. Accession Number (this is obtained from the Pathological Technology Section and this will be the number that will come back to the investigators on the finished slides). 4. Number of pieces of tissue.-5. Investigator's Experimental Number.-6. Special stains or orientation of tissues when required. (Green tags are available from us and should be used along with the white tag on the bottle to denote any request other than Routine Hematoxylin and Eosin Stain). Do Not Write on Green Tags.

(b) Request that tissues be sent on a Rush basis should be made only when absolute necessary.

(c) All tissues for processing are to be delivered to the Pathological Technology Section, Laboratory of Pathology. Investigators will be called when their slides are completed.

If information is required for other procedures, please contact Mrs. Coolidge, Head of Section, Extension 496-5951, Building 37, Room 1B23.

FIXATIVES Consult Index and Technique for Pertinent Information Before Fixation of Tissue

Fixative	Fixation Time	Prep. for Sectioning	Remarks
ABSOLUTE ALCOHOL Several changes	12-24 hours	Dehydration has already started; place in 100% alcohol.	Alcohol penetrates poorly; blocks must be thin, +3 mm.). Over-fixation makes tissues hard and brittle. Recommended for enzymes, bacteria and glycogen.
B-5 Mercuric Chloride . . . . . 6 gm Sodium Acetate (anhydrous) . . . . . 1.25 gm Distilled water (hot) . . . . . 90 ml Just before use add: 37-40% Formaldehyde . . . 10 ml	2-24 hours 2 days	Place-in-70% alcohol until processed.	Treat same as Zenker fixed tissue.
BAKER'S 10% Neutral formalin . . . 100 ml Calcium chloride . . . . . 1 gm		Frozen section or same as 10% Formalin for paraffin.	Tissues can remain in fixative indefinitely.Recommended for phospholipods.
BILSTAD (Modified Carnoy's) Absolute isopropyl alcohol . . . . . 6 parts	1-8 hours	see "Insect Processing	Recommended for worms and small insects or other chitinous material. Toluene, xylene and

Chloroform ..... 3 parts Formic acid ..... 1 part		Schedule."	high grade ethanol hardens chitin and should be avoided.
BOUIN'S (B) Saturated aqueous picric acid ..... 75 ml. 37-40% Form - aldehyde ..... 25 ml. Acetic acid ..... 5 ml.	4-18 hours	Wash in several changes	One of the best fixatives for general purpose, and also special technics, because penetration and fixation is good, and delicate detail is well preserved. Acts as a mordant for some stains. RBC destroyed, and unless thoroughly washed, will interfere with azure-eosinate stains. Cytoplasmic staining not sharp.
BOUIN - HOLLANDE (BH) Distilled water..... 100 ml Picric acid ..... 4 gm Copper acetate ..... 2.5 gm 37-40% Formaldehyde .... 10 ml Acetic acid ..... 1.5 ml	Same as B	Same as B	Originally used for gross demonstrations of areas of calcification in lymph nodes or in fat necrosis.
BUFFERED NEUTRAL FORMALIN 37-40% Formaldehyde ... 100 ml Water ..... 900 ml Sodium phosphate monobasic ..... 4 gms Sodium Phosphate dibasic (anhy) ..... 6.5 gms	Same as F	Same as F	AFIP considers this the best routine fixative. Some laboratories use 2 gms of calcium or sodium acetate in 100 ml. of 10% Formalin as neutralized formalin but this mixture is not satisfactory because it will distort tissue with excessive swelling.

FIXATIVES Consult Index and Technique for Pertinent Information Before Fixation of Tissue

Fixative	Fixation Time	Prep. for Sectioning	Remarks
CARNOY'S Absolute ethyl alcohol .... 6 parts Chloroform ..... 3 parts Acetic acid..... 1 part	1½ - 5 hours or overnight in refrigerator.	Transfer to 100% alcohol and process.	Rapid fixation, preserves glycogen, nuclear staining good. Nissl granules will preserved dehydrates as well as fixes. Destroys RBCs and acid soluble granules.
COBALT NITRATE Cobalt nitrate ..... 1 gm Distilled water ..... 100 ml. 37-40% Formaldehyde.... 15 ml.	2-4 hours	Rinse in distilled water and place in 1.5% silver nitrate solution for 16-24 hours.	For Golgi. Specimens must not exceed 1 mm. in thickness. Three specimens are fixed simultaneously.
DUBOSCQ-BRASIL (Modified Bouin's) 80% Alcohol..... 150 ml 37-40% Formaldehyde .... 60 ml Acetic acid..... 15 ml Picric acid ..... 1 gm	4-18 hours	Same as Bilstad	Same Bilstad
10% FORMALIN (F) 37-40% Formaldehyde .... 10 ml Water* ..... 90 ml	24-48 hours at room temperature	Wash out excess formalin with	*Tap water is used because it is usually less acid than distilled water. Acidic formalin is

	(Fixation time is shortened when tissues are agitated, solutions are changed, or solutions are heated. Heat hastens postmortem changes and must be used with care).	water; 5 minutes - 12 hours. May store in 70% alcohol.	better for certain silver impregnation techniques, but usually neutral, or slightly alkaline formalin is preferred. Formaldehyde will oxidize to formic acid and crystals (formalin pigment) can form in tissues when stored. 10% formalin may not harden some cytoplasmic structures adequately, but it penetrates quickly with little shrinkage, preserves blood, fats (expecially myelin in nerve fiber sheaths), amyloid, and hemosiderin. It permits most chemical reactions but dissolves glycogen, uric acid, and sodium biurate crystals.
10% FORMALIN, ALCOHOLIC Absolute alcohol..... 9 parts 37-40% Formaldehyde... 1 part	3-6 hours	Dehydration has already started; place in 100% alcohol.	Recommended for glycogen, fibrin, smears, or when injecting a dye. Penetrates quickly; starts dehydration, but removes hemoglobin, shrinks tissues and makes them brittle and difficult to cut if overfixed.
10% FORMALIN, NEUTRAL 10% Formalin..... Any amount Calcium or Magnesium carbonate..... to excess	Same as F	Same as F	Eliminates formation of formic acid and keeps large quantities of prepared formalin from becoming acid. Same advantages and disadvantes as above. May form crystals in tissue.

FIXATIVES Consult Index and Technique for Pertinent Information Before Fixation of Tissue

Fixative	Fixation Time	Prep. for Sectioning	Remarks
FORMALIN AMMONIUM BROMIDE 15% Formalin..... 100 ml Ammonium bromide ..... 2 gms	3-15 days	Frozen section	Good for Cajal gold sublimate technique for astrocytes.
FORMALIN, SALINE 10% Formalin..... 100 ml Sodium chloride..... 1 gm	Same as F	Same as F	Sodium chloride neutralizes the formalin and preserves red blood cells.
GLUTERALDEHYDE 25% Gluteraldehyde ... 10-20 ml (stored over cocoanut charcoal 10 gms/100 ml. at 4°C.) Sorenson's buffer ..... 100 ml (M/10 Anhydrous sodium phosphate dibasic ..... 4 parts	4mm. tissue 6-24 hours* smaller tissues 2-4 hours	Good for electron microscopy, histochemistry, or routine paraffin sections.	*For enzyme staining, fix in cold fixative; allow for slower penetration. There is much background staining. PAS method loses specificity, but this may be eliminated by immersing sections in a mixture of concentrated glacial acetic acid and aniline oil.
LILLIE'S AAF 37-40% Formaldehyde ... 10 ml 95% Alcohol..... 85 ml Acetic acid 5ml	4-6 hours for small tissues. 10 minues for smears.	Wash in several changes of 70% alcohol	This is a good rapid fixative for small pieces of tissue. It is an excellent fixative for tissue culture cells and thyroid colloid (less vacuolization).

ORTH'S Potassium dichromate, 2.5% aqueous solution..... 90 ml Just before use, add: 37-40% Formaldehyde ... 10 ml	36-72 hours	Wash thoroughly in running water 6-24 hours; store in 70% alcohol.	Recommended for mitotic figures, RBC, bone collodial material, chromaffin, rickettsiae, and bacteria. Myelin well preserved, hemosiderin poorly preserved. Time consuming because penetration is slow and tissue must be washed thoroughly.
REGAUD's FLUID 3% potassium dichromate. 80 ml Just before use, add: 37-40% Formaldehyde ... 20 ml	4 days, changing every day.	After fixation mordant for 7 days in 3% potassium dichromate, changing every other day. Wash in running water 24 hours.	For mitochondria. Recommended for cytoplasmic fixation. Hardens with little or no shrinkage but poor nuclear fixative and can be followed by limited number of stains. (Telleysniczky also used 3% aq. sol. of potassium dichromate, plus 5 ml. glacial acetic acid per 100 ml.)
SMITH'S 5% Potassium Dichromate ..... 8.75 ml Just before use add: 37-40% Formaldehyde .... 10 ml Acetic acid ..... 2.5 ml	8-18 hours	Wash in running water 12-24 hours. Place in 70% alcohol until processed.	Good for yolk-rich material, also produces maximum enhancement for juxtaglomerular granules.

FIXATIVES Consult Index and Technique for Pertinent Information Before Fixation of Tissue

Fixative	Fixation Time	Prep. for Sectioning	Remarks
TELLY'S 37-40% Formaldehyde ..... 5 ml 70% Ethyl alcohol ..... 100 ml Acetic acid ..... 5 ml	12-24 hours	Wash in few changes of 70% alcohol. Store in 70% alcohol.	Recommended for glycogen. Tissue cuts and stains well. (Telleysniczky also known for a potassium dichromate fixative. See Regaud's).
ZENKER base (Z) Mercuric chloride ..... 50 gm Potassium dichromate ..... 25 gm Sodium sulfate ..... 10 gm Distilled water ..... 1000 ml	6-24 hours	Wash in running water 12-24 hours. Place in 70% alcohol until processed.	All fixatives containing mercuric chloride form mercuric chlorid crystals in the tissue. Run slides through Lugol's Iodine and a Hypo solution to remove crystals before staining. Fixative penetrates poorly; blocks should be thin. Over-fixation will cause loss of affinity for nuclear stains.
ZENKER ACETIC (ZAC) Zenker base ..... 95 ml Before use add: Acetic acid ..... 5 ml	6-18 hours	Same as Z	Good nuclear stain; can be used as mordant after Formalin fixation.
ZENKER ACETIC FORMALIN (ZAF) Zenker base ..... 95 ml	6-18 hours	Same as Z	Recommended for most stains. Carmine stains

Before use, add: Acetic acid..... 5 ml 37-40% Formaldehyde ..... 5 ml			are particularly brilliant after sublimate fixatives. Good for blood-forming organs.
ZENKER FORMALIN (ZF) OR HELLY'S (H) Zenker base..... 95 ml Just before use, add: 37-40% Formaldehyde ..... 5 ml	6-18 hours	Same as Z	Same as ZAC

DECALCIFICATION

Versenate Method of Decalcification

1. Fix in 10% Formalin. Other fixatives may also be used.
2. Place in saturated solution of Versenate* until decalcification is complete.

VERSENATE SOLUTION

Versenate..... 10.0 gm.
Distilled water (pH 5.5 to 6.5)..... 100.0 ml.

3. Place in 70% alcohol. Washing is unnecessary.
4. Dehydrate, clear and infiltrate with paraffin or process as desired.

Remarks:

Most bone specimens (3 mm. thick) are decalcified in 2-4 days but specimens may be left in Versenate solution as long as 14 days without noticeable effects on the staining quality. This is especially true if the solution is between pH 5.5 and 6.5 Versenate may be used for decalcification of dense calcified materials. This, however, may require considerable time; in that event, the Versenate decalcifying solution should be changed at intervals of 3 or 4 days. The advantage of the Versenate method lies in the preservation of the staining affinity of the cells. Enzyme activity is also preserved.

Reference: Birge, E.A., and Imhoff, E.C.,: Versenate as a Decalcifying Agent for Bone. Amer. J. Clin. Path. 22: 192-193, 1952.

Formic Acid - Sodium Citrate Decalcification

Fixation:

Any well fixed tissue

Prepare: (Refrigerate as a stock solution)

Solution A

125 ml..... Formic Acid
125 ml..... Distilled Water

Solution B

50 gms..... Sodium Citrate
250 ml..... Distilled Water

Mix equal portions of Solutions A and B immediately before use. Use approximately 20 times the volume of the tissue to be decalcified. Mouse bones usually require 18-24 hours to complete decalcification. Wash in running water 18-24 hours to remove acid. The decalcified tissue can be stored in 70% alcohol or processed immediately.

Determination of End-Point of Decalcification

Prepare:

5% Ammonium Hydroxide
5% Ammonium Oxalate

After 18-24 hours of decalcification - Change solution.

After 4 hours in fresh solution -

1. Pipette 5 ml. of decalcifying fluid from the bottom of the bottle containing the tissue
2. Add 5 ml. each of the Ammonium Hydroxide and Ammonium Oxalate.
3. Mix and let stand for 15-30 minutes.
4. If solution turns cloudy the specimen is not completely decalcified. Change decalcifying solution and repeat test at 4 hour intervals until test solution remains clear.

*Versenate (Versense) is a proprietary term for ethylenediaminetetra-acetate.

STOCK SOLUTION INDEX

ACETATE BUFFER 1 M (pH 5) - ACID PHOSPHATASE
13.6% Sodium Acetate (M.W. 136.08) ..... 100 ml.
Acetic Acid, 6% ..... 50 ml.

ACETATE BUFFER (0.1 M pH 6.5)
Solution A
Sodium Acetate (M.W. 136.08) ..... 1.36 gm.
Distilled water ..... 100 ml.
Solution B
Acetic Acid ..... 0.6 ml.
Distilled water to make ..... 100 ml.
pH 6.5 Buffer
Solution A ..... 100 ml.
Solution B ..... 1.2 ml.

ACETIC ACID 0.5%

ACETIC ACID 1%

ACETIC ACID 3%

ACETIC ACID 10%

ACETIC ACID 12%

ACETIC ACID, 0.2 M
Distilled water ..... 494 ml.
Acetic Acid (M.W. 60.05) ..... 6 ml.

ACETIC ALCOHOL, 1%
Acetic Acid ..... 1.0 ml.
Alcohol, 95% ..... 99 ml.

ACETONE - PICRIC ACID
Picric Acid ..... 0.25 gm.
Acetone ..... 1 pint

ACID ALCOHOL, 1%
Hydrochloric Acid ..... 1 ml.
Alcohol, 70% ..... 99 ml.

ACID ALCOHOL, 5%
Hydrochloric Acid ..... 5 ml.
Alcohol, 70% ..... 95 ml.

ACID FUCHSIN, 1%

ACID FUCHSIN
Acid Fuchsin ..... 1.0 gm.
Distilled water ..... 100 ml.
Trichloracetic Acid ..... 3.0 gm.

ACID PHOSPHATASE SUBSTRATE SOLUTION (leukocytes)
Dissolve 30 mg. Napthol AS Phosphate* in 0.25 ml. N, N-dimethyl formamide.** Then add 100 ml. ACETATE BUFFER, (s) pH 5 (s). Stable for several months in refrigerator.

ACRIDINE ORANGE, 0.1%
Refrigerate.

ALCIAN BLUE, 1%

ALCIAN BLUE
Alcian Blue ..... 1.0 gm.
Distilled water ..... 97 ml.
Acetic Acid ..... 3.0 ml.
Adjust the pH to 2.5

ALCIAN BLUE, 1% ALCOHOLIC
Alcian Blue ..... 1.0 gm.
95% Alcohol ..... 100 ml.

ALDEHYDE FUCHSIN - GOMORI
Alcohol, 70% ..... 100 ml.
Basic Fuchsin ..... 0.5 gm.
Hydrochloric Acid ..... 1.0 ml.
Paraldehyde U.S.P. ..... 1.0 ml.
Let solution ripen until it has a deep purple color. This may take up to 36 hours. Stain will last up to 6 weeks in refrigerator. Make fresh for pituitary stains.

ALIZARIN RED S, 0.1%

ALKALINE PHOSPHATASE BUFFER SOLUTION
Sodium Barbitol ..... 5.0 gm.
Calcium Chloride ..... 5.0 gm.
Magnesium Chloride ..... 0.5 gm.
Distilled water ..... 1000 ml.

ALKALINE PHOSPHATASE SUBSTRATE SOLUTION (Leukocytes)
Dissolve 30 mg. Napthol AS Phosphate* in 0.5 ml. N, N-dimethyl formamide**. Then add 100 ml. TRIS BUFFER pH 9.1 (s).
Store in refrigerator. Stable for several months.

ANILINE-ACID FUCHSIN
Acid Fuchsin ..... 20.0 gm.
Distilled water ..... 98.0 ml.
Aniline ..... 2.0 ml.

ANILINE ALCOHOL SOLUTION
Aniline oil ..... 1 ml.
90% Alcohol ..... 1000 ml.

ANILINE BLUE, 1%

*Cyclo Chemical Corp., Los Angeles, California
**Fisher Scientific Co., Fair Lawn, New Jersey

ANILINE BLUE-MALLORY HEIDENHAIN (KNOEFF)
Aniline Blue (water soluble) ..... 0.5 gm.
Orange G ..... 2 gm.
Oxalic Acid ..... 2 gm.

Distilled water ..... 100 ml.
5% PHOSPHOTUNGSTIC ACID (s)..... 1 ml.

ANILINE BLUE-MASSON
Aniline Blue ..... 2.5 gm.
Distilled water..... 97.5 ml.
Acetic Acid ..... 2.5 ml.

ANILINE BLUE SOLUTION - HEIDENHAIN
Aniline blue, water soluble..... 0.5 gm.
Orange G..... 2.0 gm.
Distilled water ..... 100 ml.
Acetic Acid ..... 8.0 ml.

APATHY'S SYRUP
Gum Arabic (Acacia)..... 50 gm.
Sucrose (Cane Sugar)..... 50 gm.
Sodium Chloride..... 10 gm.
Distilled water ..... 100 ml.
Mix and place in 60° oven until dissolved. Restore evaporation loss with water. Add Thymol for preservative. Filter before use.

1% ASTRA BLUE SOLUTION
Astra Blue ..... 1 gm.
1% ACETIC ACID (s)..... 100 ml.

AZOCARMINE G
Azocarmine G..... 0.2 gm.
Distilled water ..... 200 ml.
Bring to boil. Filter at 56°C. Cool and add 1.0 ml. Acetic Acid. Keep in refrigerator. Filter before use.

BASIC FUCHSIN, 0.5% ALCOHOLIC
Basic Fuchsin ..... 0.5 gm.
Alcohol, 70% ..... 100 ml.

BASIC FUCHSIN SOLUTION, SATURATED AQUEOUS
Basic Fuchsin.....approximately..... 0.25 gm.
Distilled water ..... 100 ml.

BEST'S CARMINE SOLUTION
Potassium Carbonate..... 1.0 gm.
Carmine..... 2.0 gm.
Potassium Chloride ..... 5.0 gm.
Distilled water..... 60.0 ml.
Heat gently and cautiously, but do not boil. Cool. Add 20 ml. 28% ammonium hydroxide. Refrigerate. Keeps several weeks.

BORAX (See Sodium Borate)

BORIC ACID M/5 (0.2 M) (Holmes Buffer)
Boric Acid (M.W. 61.84) ..... 6.2 gm.
Distilled water ..... 500 ml.

BOUIN (s)

PICRIC ACID, SATURATED AQUEOUS (s)	75.0 ml.
37-40% Formaldehyde	25.0 ml.
Acetic Acid	5.0 ml.

BOWIE STAIN PRECIPITATE

Biebrich Scarlet	1 gm.
Dissolve in - Distilled water	250 ml.

Filter through a rapid filter paper into a 1000 ml. beaker.

Ethyl Violet	2 gm.
Distilled water	500 ml.

Dissolve and filter a small amount at a time into the Biebrich Scarlet filtrate. Use a mechanical stirrer for constant mixing. The Ethyl Violet solution is cautiously added until the end-oint of neutralization is reached. The end-point is determined when a small amount of the mixture placed on a filter paper does not show any color other than that of the precipitate itself. A colorless halo with slight peripheral coloration by the precipitate will be noted. Test the dye solution often while filtering. The end-point is a critical factor in obtaining a satisfactory dye solution. When the end-point is reached, the mixture is then filtered and the precipitate remaining on the filter paper is dried in a 37°C. oven. Store the dried precipitate in a well-stoppered bottle in the refrigerator. Makes approximately 1 gram.

BUFFERED NEUTRAL FORMALIN

37-40% Formaldehyde	100.0 ml.
Water	900.0 ml.
Sodium Phosphate, monobasic	4.0 gm.
Sodium Phosphate, dibasic (anhydrous)	6.5 gm.

CALCIUM CHLORIDE 0.1 M

Calcium Chloride (N.W. 110.99)	5.5 gm.
Distilled water	500.0 ml.

CARBOL-FUCHSIN DIMETHYLSULFOXIDE

Basic Fuchsin (pure crystalline)	4 gm.
Alcohol, 95%	25 ml.
Dissolve and add Phenol (melted)	12 gm.

Mix

Glycerin	25 ml.
Dimethylsulfoxide	25 ml.

Bring solution to 160 ml. with distilled water. Mix and let stand for 30 minutes before using. Store in amber glass or plastic bottle.

CARNOY'S

Absolute Alcohol	6 parts
Chloroform	3 parts
Acetic Acid	1 part

CELESTINE BLUE - LENDRUM

Ferric Ammonium Sulfate (ferric alum)	5.0 gm.

Distilled water ..... 100.0 ml.
Bring to boil. Add:
Celestine Blue ..... 0.5 gm.
(Boil for 3 minutes, cool and filter).
Add Glycerin ..... 14.0 ml.
Hydrochloric Acid ..... 2 ml.
Solution lasts for months.

CELLOIDIN, 1% STOCK
Parlodion ..... 5.0 gm.
Absolute Alcohol ..... 500.0 ml.
Stopper and let stand until Parlodion is dissolved.
For Use:
CELLOIDIN STOCK, 1% (s) ..... 1 part
Ether ..... 1 part
Keep tightly stoppered.

CELLOIDIN, 1% IN METHYL BENZOATE

CELLOIDIN, 3% IN METHYL BENZOATE

CHROMIC ACID, 4%

CHROMIC ACID, 5%

CITRIC ACID, M/15 in 25% METHYL ALCOHOL
Citric Acid (M.W. 210.15) ..... 7 gm.
25% Methyl Alcohol ..... 500 ml.

CITRIC ACID, M/10 (0.1 M)
Citric Acid (M.W. 210.15) ..... 10.5 gm.
Distilled water ..... 500.0 ml.

COBALT NITRATE, 2%

COLLOIDAL IRON - MÜLLER'S
Bring 250 ml. distilled water to boil. While the water is boiling add 4.4 ml. commercial FERRIC CHLORIDE (s) and stir. When solution turns dark red, remove from heat and cool. Solution keeps many months. Water must be kept boiling while Ferric Chloride is being added or results will be faulty.

CONGO RED, 1%

COOLIDGE FROZEN SECTION STAIN CLARIFIER
Prestone Antifreeze ..... 95 ml.
Tergitol. Anionic 7 ..... 5 ml.

CRESYL VIOLET ACETATE
Cresyl Violet Acetate (Cresyl Echt Violet) ..... 1 gm.
Distilled water ..... 100 ml.
Acetic Acid ..... 0.1 ml.
pH should be 3.7. Allow to stand 16-20 hours before use. Do Not Filter.
Note: The dye solution will keep several months. Deterioration of old dye solutions gives increased background staining, with a shift of color to violet. Discard solution.

CROCEIN SCARLET-ACID FUCHSIN

Solution A:

Crocein Scarlet Moo	0.1 gm.
Distilled water	99.5 ml.
Acetic Acid	0.5 ml.

Solution B:

Acid Fuchsin	0.1 gm.
Distilled water	99.5 ml.
Acetic Acid	0.5 ml.

CRYSTAL VIOLET - AMYLOID

Absolute Alcohol	10.0 ml.
Crystal Violet	1.0 gm.
Methyl Violet 2B	0.5 gm.
Distilled water	90.0 ml.

CRYSTAL VIOLET SOLUTION, 1%

CUPRIC SULFATE 0.1 M

Cupric Sulfate (M.W. 249.69)	24.97 gm.
Distilled water	1000.0 ml.

DECALCIFYING SOLUTION A, Formic Acid

Distilled water	250 ml.
Formic Acid	250 ml.

DECALCIFYING SOLUTION B, Sodium Citrate

Sodium Citrate	100.0 gm.
Distilled water	500 ml.

DIASTASE SOLVENT

Sodium Chloride	8.0 gm.
Secondary Sodium Phosphate (Na_2HPO_4)	0.282 gm.
Primary Sodium Phosphate ($NaH_2PO_4H_2O$)	1.974 gm.
Distilled water	1000.0 ml.

To use: To every 100 ml. of DIASTASE SOLVENT (s) use 0.1 gram Diastase of malt.

EOSIN - 0.5% ALCOHOLIC

Eosin Y	10.0 gm.
Dissolve in distilled water	200.0 ml.
Add Absolute Alcohol	1800.0 ml.

For Acid Eosin:
1 ml Acetic acid for a 1000 ml. staining dish.
0.5 ml. Acetic acid for a 500 ml. staining dish.

EOSIN AZURE 50 (EA 50) - Pap Stain

This stain may be obtained from Orth Diagnostics, Raritan, N.J.

EOSIN B, 1%

FAST GREEN, STOCK

Fast Green FCF	0.5 gm.
Orange G	2.0 gm.

Distilled water ..... 100.0 ml.
Bring to boil and cool to room temperature. Filter. Add 8 ml. Acetic acid.
For use dilute 1:3 with distilled water.

FERRIC CHLORIDE, 2%

FERRIC CHLORIDE, 10%

FERRIC CHLORIDE, COMMERCIAL SOLUTION 10% w/v
Purchased from Harleco Co.

FORMALIN, 2% (Sevier)
Formaldehyde (37-40%) ..... 2 ml.
Tap water ..... 98 ml.

FORMALIN, 10%
Formaldehyde (37-40%) ..... 50.0 ml.
Tap water ..... 450.0 ml.

FORMALIN, 20%

FOUCHET'S REAGENT
Trichloracetic Acid ..... 25.0 gm.
Distilled water ..... 100.0 ml.
FERRIC CHLORIDE, 10% (s) ..... 10.0 ml.

GALLEGO'S SOLUTION
Distilled water ..... 500.0 ml.
Formaldehyde 37-40% ..... 10.0 ml.
Acetic Acid ..... 5.0 ml.

GELATIN SOLUTION FOR DIPPING SLIDES
1 gram gelatin dissolved in 1000 ml. hot distilled water. Cool. Add 0.1 gram Chromium Potassium Sulfate. Refrigerate. Dip clean slides in above solution. Stand slides on end and dry. Slides may be kept in refrigerator in a dust proof box.

GIEMSA
Giemsa ..... 0.5 gm.
Glycerin ..... 33.0 ml.
Place in 60°C. oven for several hours to dissolve.
Add:
Methyl Alcohol....(acetone free) ..... 33.0 ml.
Giemsa Staining Solution may also be commercially purchased.

GLYCERIN GELATIN (MOUNTING MEDIA)
Gelatin ..... 10.0 gm.
Distilled water ..... 60.0 ml.
Heat until gelatin is dissolved. Then add:
Glycerin ..... 70.0 ml.
Store in refrigerator and warm before using.

GOLD CHLORIDE, 1%
Wash a 15 grain vial of Gold Chloride (yellow). Score vial with a diamond pencil or glass tubing cutter. Break vial carefully and drop

into an empty bottle. Add 100 ml. distilled water and shake bottle. Refrigerate.

GOLD CHLORIDE, 0.1% Refrigerate.

GOLD CHLORIDE, 0.2% Refrigerate.

GOLD CHLORIDE, 0.5%. Refrigerate.

GOLD CHLORIDE, AZZOPARDI MODIFICATION

Gold Chloride	1 vial
Distilled water	100 ml.

Add 3 drops Acetic acid. Refrigerate.

GOODPASTURE'S SOLUTION

Basic Fuchsin	0.59 gm.
Aniline (Do not pipette)	1.0 ml.
Phenol crystals (melted)	1.0 ml.
30% Alcohol	100.0 ml.

(Ziehl's carbol fuchsin diluted 1:4 with distilled water is a satisfactory substitute).

HEMATOXYLIN 10%, ALCOHOLIC

Hematoxylin	10.0 gm.
Absolute Alcohol	100.0 ml.

This hematoxylin should ripen for 6 months before use.

HEMATOXYLIN 5%, ALCOHOLIC

Hematoxylin	5.0 gm.
Absolute Alcohol	100.0 ml.

HEMATOXYLIN, CLARA'S

Solution A

Distilled water	100.0 ml.
Potassium Dichromate	3.2 gm.
Chrome Alum	1.6 gm.
Chromic Acid	0.125 gm.
Ammonium Molybdate	0.25 gm.

Solution B

Distilled water	98.0 ml.
Acetic Acid	2.0 ml.
Hematoxylin	1.0 gm.

HEMATOXYLIN - GOMORI - CHROME ALUM

Distilled water	100.0 ml.
Chrome Alum	1.5 gm.
Hematoxylin	0.5 gm.

Dissolve the alum and the dye each in 50 ml. of the distilled water. Mix solutions together and add:

POTASSIUM DICHROMATE, 5% (s)	2.0 ml.
2.5% Sulfuric Acid	2.0 ml.

Allow 2 days to ripen.

HEMATOXYLIN, HARRIS

Hematoxylin crystals ..... 5.0 gm.
Alcohol, Absolute ..... 50.0 ml.
Ammonium or Potassium Aluminum Sulfate ..... 100.0 gm.
Distilled water ..... 1000.0 ml.
Mercuric Oxide (red) ..... 2.5 gm.
Dissolve hematoxylin in alcohol, the alum in water by aid of heat. Remove from heat and mix two solutions. Bring rapidly to a boil. Remove from heat, add mercuric oxide slowly. Reheat until dark purple. Remove from heat and place vessel in a pan of cold water until cool. Before use: Add 2 ml. Acetic acid to 100 ml. stain. Filter.

HEMATOXYLIN, MAYER'S

Hematoxylin crystals ..... 4.0 gm.
Distilled water ..... 2000.0 ml.
Sodium Iodate ..... 0.8 gm.
Potassium Aluminum Sulfate (alum) ..... 200.0 gm.
Citric Acid 4.0
Chloral Hydrate ..... 200.0 gm.
Dissolve the alum in water, without heat; add and dissolve the hematoxylin in this solution. (Shake well). Then add the sodium iodate (shake well), citric acid, (shake well) and the chloral hydrate. Shake until all components are in complete solution. The final color of the stain is reddish violet. Can be used immediately, but improves after 1 week.
Note: If Potassium Aluminum Sulfate is in big crystal form, crush with mortar and pestle.

HEMATOXYLIN-PHOSPHOTUNGSTIC ACID (PTAH)

Hematoxylin ..... 1.0 gm.
Phosphotungstic Acid ..... 2.0 gm.
Distilled water ..... 1000.0 ml.
Dissolve hematoxylin and phosphotungstic acid in separate portions of distilled water. Because hematoxylin dissolves very slowly at room temperature, heat the solution until no more color change is noted. Let hematoxylin solution cool and then combine with phosphotungstic acid solution.
Add: Potassium Permanganate ..... 0.177 gm.
Allow at least 6 weeks to ripen.

HEMATOXYLIN, REGAUD'S

Hematoxylin ..... 1.0 gm.
Alcohol, 95% ..... 10.0 ml.
Glycerin ..... 10.0 ml.
Distilled water ..... 80.0 ml.
Allow several weeks for ripening.

HEMATOXYLIN, WEIGERT'S IRON

Solution A:
Hematoxylin crystals ..... 1.0 gm.
95% Alcohol ..... 100.0 ml.

Solution B:

Ferric Chloride, 29%	4.0 ml.
Distilled water	95.0 ml.
Hydrochloric Acid	1.0 ml.

HOLMES BUFFERS

See: SODIUM BORATE, M/20 (s) and BORIC ACID M/5 (s)
See: Lillie Buffer Tables for pH determination

HYDROBROMIC ACID SOLUTION

Hydrobromic Acid	20.0 ml.
Distilled water	60.0 ml.

HYDROCHLORIC ACID, 1%

HYDROCHLORIC ACID, N/10 (0.1N)

Distilled water	495.83 ml.
Hydrochloric Acid (Sp. gr. 1.19)	4.17 ml.

HYDROCHLORIC ACID, NORMAL

Distilled water	916.5 ml.
Hydrochloric Acid....(Sp. gr. 1.19)	83.5 ml.

HYDROCHLORIC ACID, 3 NORMAL

Distilled water	374.75 ml.
Hydrochloric Acid (Sp. gr. 1.19)	125.25 ml.

HYDROCHLORIC ACID, 5 NORMAL

Distilled water	291.25 ml.
Hydrochloric Acid (Sp. gr. 1.19)	208.75 ml.

HYPO

See: SODIUM THIOSULFATE

IODINE - GRAM'S IODINE SOLUTION

Iodine	1.0 gm.
Potassium Iodide	2.0 gm.
Distilled water	300.0 ml.

IODINE - LUGOL'S SOLUTION

Potassium Iodide	6.0 gm.
Iodine	4.0 gm.
Distilled water	100.0 ml.

IODINE - LUGOL'S, WEIGERT'S MODIFICATION

Iodine	1.0 gm.
Potassium Iodide	2.0 gm.
Distilled water	100.0 ml.

IODINE SOLUTION, 0.5% (H & E)

Iodine	10.0 gm.
Dissolve in Absolute Alcohol	1400.0 ml.
Distilled water	600.0 ml.

IODINE, TINCTURE (Stock)

Iodine	7.5 gm.

Potassium Iodide ..... 5.0 gm.
Distilled water..... 5.0 ml.
Alcohol, 95%, sufficient to make ..... 100.0 ml.

IODINE - VERHOEFF'S
Iodine ..... 2.0 gm.
Potassium Iodide ..... 4.0 gm.
Distilled water..... 100.0 ml.

KERNECHTROT
See: NUCLEAR FAST RED (s)

KINYOUN'S CARBOL FUCHSIN
Basic Fuchsin ..... 4.0 gm.
Phenol crystals, melted ..... 8.0 ml.
Alcohol, 95%..... 20.0 ml.
Distilled water..... 100.0 ml.
Filter before use.

LEAD NITRATE, 5%

LEUCO PATENT BLUE SOLUTION
Patent Blue V (Alphazurine 2G) ..... 1.0 gm.
Distilled water..... 100.0 ml.
Zinc, powdered ..... 10.0 gm.
Acetic Acid ..... 2.0 ml.
Boil until colorless. Cool and filter. Keep refrigerated in a tightly stoppered bottle.

LIGHT GREEN 0.1%
Light green ..... 0.1 gm.
Distilled water..... 100.0 ml.
Acetic Acid ..... 1.0 ml.
For use dilute 1:3 with distilled water.

LITHIUM CARBONATE, 0.05%

LITHIUM CARBONATE, SATURATED AQUEOUS

LUXOL FAST BLUE SOLUTION
Luxol Fast Blue MBS..... 0.1 gm.
Alcohol, 95% ..... 100.0 ml.
Dissolve dye in alcohol. Add: .5 ml. 10% Acetic acid.
Solution is stable.

MALACHITE-GLYCERIN-ACID SOLUTION (combined decolorizer and counterstain).
Mix in the following order -
2% Aqueous Solution Malachite Green (96% dye content) 220 ml.
99.5% Acetic Acid ..... 30 ml.
Glycerin ..... 50 ml.
Store at room temperature in a well-stoppered bottle.

MALLORY STAINING SOLUTION (RAPID)
Phosphotungstic Acid Crystals..... 1.0 gm.

Orange G ..... 2.0 gm.
Aniline Blue, W.S. ..... 1.0 gm.
Acid Fuchsin ..... 3.0 gm.
Distilled water ..... 200.0 ml.

MAY-GRUNWALD
May-Grunwald ..... 1.0 gm.
Methyl Alcohol ..... 400.0 ml.
Cautiously heat the alcohol to 50°C. Slowly add May-Grunwald powder. Let stand 24 hours. Filter and store in dark bottle.

McJUNKIN-HADEN BUFFER, PH 6.4
Monobasic Potassium Phosphate ..... 6.63 gm.
Anhydrous Dibasic Sodium Phosphate ..... 2.56 gm.
Distilled water ..... 1000.0 ml.

METANIL YELLOW SOLUTION
Metanil Yellow ..... 0.25 gm.
Distilled water ..... 100.0 ml.
Acetic Acid ..... 0.25 ml.

METHENAMINE-SILVER SOLUTION
Dissolve:
Methenamine ..... 3.0 gm.
Distilled water ..... 100.0 ml.
Add:
SILVER NITRATE, 5% (s) ..... 5.0 ml.
Refrigerate. Store in dark bottle.

METHYL GREEN-PYRONIN pH 5.3
Pyronin Y ..... 1.0 gm.
Methyl Green ..... 3.0 gm.
Prepare Phosphate Buffer pH 5.7 and add the dyes.
SODIUM PHOSPHATE M/5 (MGP only) (s) ..... 230.0 ml.
CITRIC ACID M/10 (s) ..... 170.0 ml.
Start with Phosphate Buffer pH 5.7 because the Methyl Green and Pyronin Y lower the pH 0.4.
The final pH must be 5.3. Refrigerate.

METHYL GREEN-PYRONIN, ROGER'S
Distilled water ..... 320 ml.
Glycerin ..... 80 ml.
Mix well.
Add each of the following ingredients and shake well after each addition.
Methyl Green ..... 0.8 gm.
Pyronin Y ..... 0.8 gm.
Liquid Phenol ..... 1.0 ml.
TRIS BUFFER pH 9.1 (s) ..... 8.4 ml.
Shake vigorously and allow solution to stand for 2 hours.
Add:
McILVAINE'S BUFFER pH 5.5 (s) ..... 3.2 ml.
Shake well and allow to stand for 1 hour. Refrigerate. (see Pearse's buffer tables)

METHYLENE BLUE (STOCK)

Methylene Blue	1.4 gm.
Alcohol, 95%	100.0 ml.

Working Solution:

METHYLENE BLUE (s)	10.0 ml.
Tap water	90.0 ml.

MUCICARMINE SOLUTION

Carmine	1.0 gm.
Anhydrous Aluminum Chloride	0.5 gm.
Distilled water	2.0 ml.

Mix stain in small test tube. Heat gently with flame for 2 minutes. Liquid becomes almost black and syrupy. Pour into 100 ml. of 70% alcohol and let stand 24 hours. Filter and use full strength.

MYELOPEROXIDASE INCUBATION MIXTURE

30% Ethyl Alcohol	100.0 ml.
Benzidine dihydrochloride	0.3 gm.
0.132 M (3.8% w/v) Zinc Sulfate ($ZnSO_47H_20$)	1.0 ml.
Sodium Acetate ($NaC_2H_3023H_20$)	1.0 gm.
3% Hydrogen Peroxide	0.7 ml.
1 N Sodium Hydroxide	1.5 ml.
Safranin O	0.2 gm.

These reagents should be added in the order listed, mixing well with each addition. The benzidine salt may contain a small amount of inert residue which will not go into solution. A precipitate forms upon addition of the remaining reagents. Omit the Safranin O if a nuclear counterstain is not desired. The final pH is 6.0. The solution should be filtered and stored in a capped bottle at room temperature. Keeps approximately 3 months.

NEUTRAL FORMALIN, 10%

10% FORMALIN (s)	any amount
Calcium or Magnesium Carbonate	to excess

NEUTRAL RED SOLUTION, 0.1%

NEUTRAL RED SOLUTION, 1%

NILE BLUE SULFATE SOLUTION, 1.5%

NUCLEAR FAST RED (KERNECHTROT)

Dissolve 0.1 gm. Nuclear Fast Red in 100 ml. of 5% Aluminum Sulfate with the aid of heat. Cool and filter.

OIL RED O SOLUTION

Oil Red O	0.5 gm.
Isopropyl Alcohol	100.0 ml.

ORANGE G-ANILINE BLUE - QUAY - GOMORI

Distilled water	100 ml.
Orange G	2 gm.
Phosphomolybdic Acid	1 gm.

Aniline Blue ..... 0.5 gm.

ORANGE G SOLUTION
Orange G ..... 2.0 gm.
PHOSPHOTUNGSTIC ACID, 5% (s)..... 100.0 ml.
Let stand 24 hours and use the supernatant.

ORANGE G SOLUTION (KREYBERG'S)
Orange G ..... 0.5 gm.
Phosphotungstic Acid ..... 2.0 gm.
Distilled water..... 100.0 ml.

ORANGE G6 (OG6) (Pap Stain)
Orth's Commercial Stain can be used or stain may be made.
Orange G6 ..... 0.5 gm.
Alcohol, 95% ..... 100.0 ml.
Add:
Phosphotungstic Acid..... 0.015 gm.

ORCEIN SOLUTION
Orcein ..... 1.0 gm.
Alcohol, 70% ..... 100.0 ml.
Hydrochloric Acid ..... 1.0 ml.
Filter before using.

OXALIC ACID, 5%

PERIODIC ACID, 0.5%

PERIODIC ACID, 1%

PHLOXINE-LENDRUM
Phloxine..... 1.0 gm.
Alcohol, 70% ..... 200.0 ml.
Calcium Chloride..... 1.0 gm.

PHLOXINE B, 1%

PHLOXINE B SOLUTION pH 3.7 (Hemoglobin & Hemosiderin)
Phloxine B ..... 5.0 gm.
Methyl Alcohol..... 80.0 ml.
Acetic Acid ..... 30.0 ml.
Adjust pH to 3.7. DO NOT FILTER BEFORE USE.

PHOSPHATE BUFFER pH 6
Sodium Phosphate (Na_2HPO_4)..... 1.26 gm.
Sodium Phosphate ($NaH_2PO_4H_2O$) ..... 8.98 gm.
Distilled water..... 1000.0 ml.
Check pH with meter. Refrigerate.

PHOSPHOMOLYBDIC ACID, 1%

PHOSPHOTUNGSTIC ACID, 1%

PHOSPHOTUNGSTIC ACID, 5%

PICRIC ACID - ACETONE

Picric Acid ..... 0.1 gm.
Acetone ..... 100.0 ml.

PICRIC ACID - SATURATED - ALCOHOLIC (95%)

PICRIC ACID - SATURATED AQUEOUS

PICRIC ALCOHOL
PICRIC ACID, SATURATED ALCOHOLIC (s) ..... 2 parts
Alcohol, 95% ..... 1 part

PICRO-ORANGE
Picric Acid Saturated in 80% alcohol ..... 100 ml.
Orange G ..... 0.2 gm.

PONCEAU RED
Ponceau de Xylidine..... 1.0 gm.
Distilled water..... 100.0 ml.
Acetic Acid ..... 1.0 ml.

POTASSIUM DICHROMATE, 5%

POTASSIUM METABISULFITE, 2%

POTASSIUM PERMANGANATE, 0.25%

POTASSIUM PERMANGANATE, 0.5%

POTASSIUM PERMANGANATE SOLUTION
Potassium Permanganate ..... 0.3 gm.
Distilled water..... 100.0 ml.
Sulphuric Acid ..... 0.3 ml.

POTASSIUM PHOSPHATE M/15 - SORENSON'S BUFFER
Potassium Phosphate (M.W. 136.09) ..... 0.941 gm.
Distilled water..... 100.0 ml.

POTASSIUM PHOSPHATE, M/10 (0.1 M) MONOBASIC
Potassium Phosphate (M.W. 136.09)..... 13.61 gm.
Distilled water..... 1000.0 ml.

SAFRAN, ALCOHOLIC, 6%
Safran du Gatinais..... 6.0 gm.
Absolute Alcohol ..... 100.0 ml.
Keep solution in an incubator at 58°C. for 48 hours. Shake frequently. Filter before use. May be reused. Store in tightly closed, dark bottle.

SAFRAN DU GATINAIS, 3.5%
Safran du Gatinais..... 14 gm.
Absolute Alcohol..... 400 ml.
Keep solution in an incubator at 58°C. for 48 hours. Shake frequently. Filter before use. May be reused. Store in a tightly closed, dark bottle. (Spice saffron may be substituted for economy. Grind spice saffron in a mortar and prepare staining solution in the same way as the Safran du Gatinais Solution.)

SAFRANIN O

Safranin O..... 0.1 gm.
0.1% Acetic Acid..... 100.0 ml.

SALINE 0.9%
Sodium Chloride..... 9 gm.
Distilled water..... 1000 ml.

SCHIFF'S REAGENT
Boil 200 ml. distilled water.
Add: Basic Fuchsin (stir)..... 1.0 gm.
Cool to 50°C. Filter.
Add: NORMAL HYDROCHLORIC ACID (s)..... 20.0 ml.
Cool to 25°C.
Add: Sodium meta-Bisulfite ($Na_2S_2O_5$)..... 1.0 gm.
Shake vigorously. Put in a dark place. Reagent takes about 2 days to turn straw color, then it is ready for use. Add charcoal, shake, and filter if the color doesn't change. Refrigerate, but bring to room temperature before use.
Stain may be removed from clothes with ACID ALCOHOL, 1%.
Test for SCHIFF'S REAGENT. Pour a few drops of SCHIFF'S REAGENT (s) into 10 ml. of 37-40% Formaldehyde. If the solution turns reddish-purple rapidly it is good. If the reaction is delayed and the resulting color is deep blue-purple, the reagent is breaking down.

SILVER NITRATE, 1%. Refrigerate.

SILVER NITRATE, 5%. Refrigerate.

SILVER NITRATE, 10%. Refrigerate.

SILVER NITRATE, 20%. Refrigerate.

SIRIUS RED (Llewellyn's)
Sirius Red F3B..... 0.5 gm.
Distilled water..... 45.0 ml.
After dye is completely dissolved, add:
Absolute Alcohol..... 50.0 ml.
1% SODIUM HYDROXIDE (s)..... 1.0 ml.
While shaking vigorously add sufficient 20% SODIUM CHLORIDE (s) to just produce a fine precipitate (up to 4 ml.). Let stand overnight before filtering. Solution is stable.

SIRIUS RED SOLUTION
Sirius Red F3BA..... 1.0 gm.
Distilled water..... 100.0 ml.
Sodium Chloride..... 0.5 gm.
Let stand overnight. Do NOT filter.

SODIUM ACETATE M/5 (0.2 M)
Sodium Acetate (MW 136 136.08)..... 2.72 gm.
Distilled water..... 100 ml.

SODIUM BARBITOL M/10 (0.1 M) (Veronal Buffer)
Sodium Barbitol (M.W. 206.183)..... 10.31 gm.

Distilled water..... 500.0 ml.

SODIUM BICARBONATE, 5%

SODIUM BISULFITE, 1%

SODIUM BISULFITE, 3%

SODIUM BISULFITE, 1M
Sodium Bisulfite (M.W. 104.07)..... 104.0 gm.
Distilled water..... 1000.0 ml.

SODIUM BORATE (BORAX), 5%

SODIUM BORATE M/20 (.05 M) (Holmes Buffer)
Sodium Borate (M.W. 201.27)..... 9.5 gm.
Distilled water..... 500.0 ml.

SODIUM BORATE 0.1M pH 9.0 (Sirius Red Amyloid)
Sodium Borate (M.W. 201.27)..... 19.072 gm.
Distilled water..... 500.0 ml.

SODIUM BORATE - SATURATED 80% ALCOHOLIC

SODIUM CARBONATE SOLUTION (Sevier)
Hydrated Sodium Carbonate ($Na_2CO_3.10H_2O$)..... 8.0 gm.
Distilled water..... 30.0 ml.

SODIUM CARBONATE, 20%

SODIUM CHLORIDE, 20%

SODIUM HYDROXIDE, 1%

SODIUM HYDROXIDE, 3.1%

SODIUM NITRITE, 1%

SODIUM NITRITE, 5%

SODIUM PHOSPHATE BUFFER M/15 (Sorenson's Buffer)
Sodium Phosphate (dibasic) (M.W. 141.98)..... 2.39 gm.
Distilled water..... 100.0 ml.

SODIUM PHOSPHATE, DIBASIC M/15 IN 25% METHYL ALCOHOL
Sodium Phosphate, dibasic (M.W. 141.98)..... 4.73 gm.
25% Methyl Alcohol..... 500.0 ml.

SODIUM PHOSPHATE, M/10 (0.1 M)
Sodium Phosphate, Dibasic (M.W. 141.98)..... 14.20 gm.
Distilled water..... 1000.0 ml.

SODIUM PHOSPHATE M/5 (0.2 M)
Sodium Phosphate, Dibasic (M.W. 141.98)..... 14.18 gm.
Distilled water..... 500.0 ml.

SODIUM PHOSPHATE M/5 - MGP ONLY
Sodium Phosphate, Dibasic ($Na_2HPO_47H_2O$)
(M.W. 268.077)..... 26.8 gm.
Distilled water..... 500.0 ml.

SODIUM SULFATE, 1%

SODIUM SULFATE, 2%

SODIUM THIOSULFATE, 2%

SODIUM THIOSULFATE, 3%

SODIUM THIOSULFATE, 5%

SORENSON'S BUFFERS

SODIUM PHOSPHATE, DIBASIC M/15 and
POTASSIUM PHOSPHATE M/15

See: Lillie Buffer Tables for pH determination.

SULFUROUS ACID RINSE, 10% (Sodium Bisulfite)

Sodium Bisulfite ($NaHSO_3$)	10.0 gm.
Distilled water	100.0 ml.

Working Solution:

SULFUROUS ACID RINSE, 10% (s)	5.0 ml.
Distilled water	95.0 ml.

Solution is too old when it loses the strong sulfur odor. Refrigerate.

TANNIC ACID, 5%

Must be made at least 48 hours in advance.

TARTRAZINE-LENDRUM

This is a saturated solution of Tartrazine in Methyl Cellosolve.
Methyl Cellosolve is the same as Ethylene Glycol Monomethyl Ether.

TELLY'S

37-40% Formaldehyde	5.0 ml.
Alcohol, 70%	100.0 ml.
Acetic Acid	5.0 ml.

TOLUIDINE BLUE

Toluidine Blue O	0.2 gm.
60% Alcohol	100 ml.

TRICHROME STAIN - GOMORI

Chromotrope 2R	0.6 gm.
Light Green	0.3 gm.
Acetic Acid	1.0 ml.
Phosphotungstic Acid	0.8 gm.
Distilled water	100.0 ml.

Aniline Blue may be substituted for Light Green if it is desirable to have collagen stain blue.

TRIS BUFFER STOCK SOLUTION

Trihydroxymethylaminimethane.(0.2M) (M.W. 121.14)	24.228 gm.
Distilled water	1000.0 ml.

Tris Buffer pH 7.3 Working Solution

TRIS BUFFER STOCK SOLUTION (s)	10.0 ml.
HYDROCHLORIC ACID, (N/10) (s)	17.0 ml.
Distilled water	13.0 ml.

Tris Buffer pH 9.1 Working Solution
TRIS BUFFER STOCK SOLUTION (s) ..... 10.0 ml.
HYDROCHLORIC ACID, (N/10) (s) ..... 2.0 ml.
Distilled water ..... 28.0 ml.
See Lillis's Third Edition, page 667, for other pH determinations.

URANIUM NITRATE, 1%

VAN GIESON
ACID FUCHSIN, 1% (s) ..... 5.0 ml.
PICRIC ACID, SATURATED AQUEOUS (s) ..... 100.0 ml.

VERONAL BUFFER
See SODIUM BARBITOL, M/10.

WALPOLE ACETATE BUFFER 0.2 M (pH 5.2)
SODIUM ACETATE 0.2 M (s) ..... 39.5 ml.
ACETIC ACID 0.2 M (s) ..... 10.5 ml.
Diluted to a total volume of 100 ml.

WATER SOLUBLE MOUNTING MEDIUM (see Apathy's Syrup)

WILDER'S-REDUCING SOLUTION
Distilled water ..... 50.0 ml.
Formaldehyde (37-40%) ..... 0.5 ml.
URANIUM NITRATE, 1% (s) ..... 1.5 ml.

WRIGHT'S STAIN
Wright's ..... 1.0 gm.
Methyl Alcohol ..... 600.0 ml.
Use unopened bottle of methyl alcohol. USP. Grind dye in clean, dry mortar. Rinse mortar and pestle with methanol to harvest all of dye. Filter before use. Store in dark bottle. Stable one year.

ZENKER BASE
Mercuric Chloride ..... 50.0 gm.
Potassium Dichromate ..... 25.0 gm.
Sodium Sulfate ..... 10.0 gm.
Distilled water ..... 1000.0 ml.

ZIEHL-NEELSEN CARBOL FUCHSIN SOLUTION
Phenol crystals, melted ..... 2.5 ml.
Absolute Alcohol ..... 5.0 ml.
Basic Fuchsin ..... 0.5 gm.
Distilled water ..... 50.0 ml.
Filter before use.

ACID FAST - Fite

Fixation:

10% Formalin preferred. Others May be used.

Technique:

Cut paraffin sections at 5-6 microns. Use control slide.

Prepare:

Xylene-Peanut Oil Solution

Peanut Oil ..... 1 part
Xylene ..... 2 parts

1% Sulfuric Acid Solution

Staining Procedure:

1. Deparaffinize in 2 changes of Zylene-Peanut Oil solution for 12 minutes each.
2. Drain, wipe off excess oil and blot.
3. Stain sections in ZIEHL-NEELSEN CARBOL FUCHSIN SOLUTION(s) for 30 minutes.
4. Wash in running water for 3 minutes.
5. Differentiate in 1% Sulfuric Acid solution until sections are pale pink, one minute.
6. Wash in running water for - 3 minutes.
7. Stain lightly with MAYER'S HEMATOXYLIN (s) for 30 seconds.
8. Blue in running water for 10 minutes.
9. Blot and allow to air dry.
10. Dip in xylene and mount.

Results:

Acid fast bacilli - red
Nocardia filaments - red
Lepra bacilli - red
Nuclei - blue

Reference: Fite, G. L., Cambre, P.J. and Turner, N.J.,: Arch. Path. 43: 624-625, 1947.

ACID FAST - FLUORESCENT - Truant

Fixation:

10% Formalin. Other general fixatives may be used.

Technique:

Cut paraffin sections at 4-5 microns. Use control slide.

Prepare:

Weigert's Working Solution

Equal parts of WEIGERT'S IRON HEMATOXYLIN (s) Solution A and Solution B.

Auramine-Rhodamine Solution

Auramine	1.5 gm.
Rhodamine B	0.75 gm.
Glycerin	75 ml.
Phenol crystals (melted)	10 ml.
Distilled water	50 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in Weigert's Working Solution for ..... 10 minutes.
3. Wash in running water for ..... 10 minutes.
4. Stain sections with Auramine-Rhodamine Solution at 60°C for 10 minutes.
5. Wash in running water for ..... 20-30 seconds.
6. Differentiate in ACID ALCOHOL, 1% (s) to remove excess stain.
7. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid fast bacilli will fluoresce reddish yellow. The Weigert staining acts as a quenching agent for the background.

Reference: Truant, J.P.: Henry Ford Hospital Med. Bull. 10: 287, 1962.

ACID FAST - Kinyoun - Modification

Fixation:

10% Formalin preferred, others may be used.

Technique:

Paraffin sections cut at 4 to 6 microns. Use control slide .

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in filtered KINYOUN'S CARBOL FUCHSIN Solution (s) for 1 hour at room temperature.
3. Wash well in tap water for 15 minutes.
4. Differentiate in 1 change of 5% ACID ALCOHOL (s) and 2 changes of 1% ACID ALCOHOL (s) until tissue is pale.
5. Wash well in tap water for 15 minutes.
6. Counterstain in MAYER'S HEMATOXYLIN (s) for 45 seconds.
7. Blue in running tap water.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid-fast bacteria - bright red
Nuclei - blue

Reference: Kinyoun, J.J.: Amer. J. Pub. Health, 5: 867, 1915.

ACID FAST - Night Blue

Fixation:
Any

Technique:
Cut paraffin sections at 6 microns. Use control slide.

Prepare:

Night Blue Staining Solution

Night Blue (C.I. 44085)	0.5 gm.
Ethanol, 95%	10 ml.
Dissolve Night Blue in the alcohol, then add -	
Distilled water	90 ml.

Filter before use. Good for 2 weeks.

9% Nitric Acid

Safranin O Working Solution

SAFRANIN O (s)	10 ml.
Distilled water	90 ml.

Staining Procedure:

1. Deparaffinize in 3 changes of xylene for 2 minutes each. Remove mercuric chloride from tissue if needed, return sections to absolute alcohol and into two changes of xylene.
2. Air dry slides.
3. Place in 2% SODIUM SULFATE (s) for 10 seconds. Wipe slide dry except where there is tissue. Do not blot, keep fair amount of solution on tissue.
4. Place in Night Blue Staining Solution for 60 minutes at 60°C.
5. Cool to room temperature, (about 30 minutes)
6. Dip once in distilled water.
7. Place in 9% Nitric Acid for 15 seconds. (Blue turns black)
8. Place in distilled water for 1 minute or longer. (Blue color returns here)
9. Decolorize in 3 changes of 70% alcohol. Check for complete decolorization with microscope. Cartilage does not decolorize well.
10. Rinse well in distilled water. Time not a crucial factor here.
11. Place in Safranin O Working Solution for 10-15 seconds.
12. Rinse in distilled water.
13. Blot and air dry - or dehydrate through 2 changes of 95% alcohol, 2 changes of absolute alcohol, clear in xylene and mount.

Results:
Acid Fast material - blue
Background - pink

Reference: Hallberg, W.: Acta Med., Scand. Suppl., 180, 194 6.

ACID FAST - 3 Minute

Fixation:

10% Formalin or any well fixed tissue.

Technique:

Cut paraffin sections at 4-6 microns. Use control slide .

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain sections in freshly filtered CARBOL-FUCHSIN-DIMETHYLSULFOXIDE Solution(s) for 3 minutes (2 minutes for smears).
3. Wash in tap water.
4. Counterstain and decolorize in MALACHITE-GLYCERIN-ACID Solution (s) for 2 minutes.
5. Wash in water.
6. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid fast bacilli - red
Background - green

Reference: Pottz, G.E. etal., Am. J. Clin, Path. 42: 552, 1964

ACID HEMATEIN - Baker's

Fixation:

Baker's Calcium Chloride Formalin. Do not transfer to alcohol.

Technique:

Cut frozen sections at 10 microns. Free floating sections may be used or sections may be mounted on GELATINIZED SLIDES (s), drained, blotted, dried, and fixed in formalin vapor for 10 minutes. Include a positive control section.

Prepare:

Potassium Dichromate - Calcium Chloride Solution

Potassium Dichromate	5 gm.
Calcium Chloride	1 gm.
Distilled water	100 ml.

Acid Hematein Solution

Hematoxylin	0.05 gm.
Distilled water	48 ml.
1% Sodium Iodate	1 ml.

Boil, allow to cool, and add:

Acetic acid	1 ml.

Filter before use.

Differentiating Solution

5% SODIUM BORATE (s)	2 ml.
Distilled water	38 ml.
Potassium Ferricyanide	0.1 gm.

Staining Procedure:

1. Place slides in Potassium Dichromate-Calcium Chloride Solution overnight.
2. Change slides to fresh Potassium Dichromate-Calcium Chloride Solution. Place in a 60°C. oven for 4 hours.
3.. Rinse slides in several changes of distilled water for 5 minutes.
4. Stain in Acid Hematein Solution at 37°C. for 5 hours.
5. Rinse in distilled water.
6. Place slides in Differentiating Solution at 37°C. for 18 hours.
7. Rinse gently in distilled water. Sections may be loose.
8. Mount in melted GLYCERIN-JELLY (s).

Results:

Phospholipids (lecithin, cephalin, sphingomyelin) - dark blue to black.

Mucin - dark blue to pale blue or brown.

Reference: Baker, Quart. J. Micr. Sci., 87: 441, 1946.

ACID PHOSPHATASE - Burstone

Fixation:
Cold Acetone

Technique:
Cut unfixed tissue on the cryostat at 6 microns, mount on slides and allow to air dry before fixation. Source of positive control - prostate.

Prepare:
Azo Dye Solution

Dissolve:
5 mg. of Napthol AS-BI, AS-CL, AS-AN, or AS-TR phosphate in 0.25 ml. of DMF (dimethylformamide).
Add:
Distilled water..... 20 ml.
0.2 M WALPOLE ACETATE BUFFER (pH 5.2) (s)..... 20 ml.
Red-Violet LB..... 30 mg.
Add:
10% Magnesium Chloride..... 2 drops
Stir. Filter into coplin jar and use immediately.

Staining Procedure:
1. Fix frozen sections in cold acetone briefly. (30 seconds).
2. Rinse quickly in distilled water.
3. Place slides in the Azo Dye Solution at 37° C. for 1/2 - 2 hours. Check under microscope for reaction.
4. Wash in running water for 5 minutes.
5. Counterstain in MAYER'S HEMATOXYLIN (s) for 1-1/2 minutes.
6. Wash in running water for 10 minutes.
7. Mount in melted GLYCERIN-GELATIN (s).

Results:
Sites of acid phosphatase activity - red
Nuclei - blue

Reference: Bowling, M.C., Histopathology Laboratory Procedures, p. 42.

ACID PHOSPHATASE - Gomori (Modified)

Fixation:

Cold 10% Formalin or Cold Acetone for 24 hours.

For Paraffin Blocks:
95% alcohol - 8 hours
100% alcohol - overnight
Chloroform I - 1 hour
Chloroform II - 1 hour
Chloroform - 3 parts)
Paraffin - 1 part) at 56°C. - 1 hour)
Paraffin I - 1 hour
Paraffin II - 1 hour
Embed at not over 56°C.

Technique:

Cut 6-10 micron sections on the Cryostat (if unfixed). When spreading paraffin sections warming tray must not be over 37°C. Do not use egg albumin on slides. Cut 8 slides (4 for control).

Prepare:

Lead Nitrate - Sodium Glycerophosphate Solution

1 M ACETATE BUFFER pH 5 (s) ..... 9 ml.
5% LEAD NITRATE (s)..... 3 ml.
Distilled water..... 18 ml.
Slowly add, while stirring
2% Sodium Beta Glycerophosphate (make fresh) ..... 9 ml.
Shake well, let stand in refrigerator for two hours. Before use add -
Distilled water ..... 100 ml.
For the Control Slides - make an equal amount of the above solutions except substitute 9 ml. of distilled water for the 2% Sodium Beta Glycerophosphate.
Note - Solutions are kept refrigerated. Acid cleaned glassware must be used. If solutions turn milky, discard and start again with clean glassware and fresh solutions. Use a ventilating hood on Step 7 of the Staining Procedure. Avoid any contact with metal.

Staining Procedure:

1. Deparaffinize slides and hydrate to water. (Use fresh solutions).
2. Incubate 4 test slides at 37°C. in the Lead Nitrate-Sodium Glycerophosphate solution for 1 1/2 - 48 hours. (For prostatic tissue -1 1/2 hours). Incubate 4 control slides in control solution for 1 1/2 - 48 hours.
3. Remove one test slide and one control slide at the following times - 1 1/2 hours, 6 hours, 24 hours and 48 hours.
4. Rinse slides in 3 changes of distilled water.
5. Rinse in 3% ACETIC ACID (s).
6. Rinse in distilled water.
7. Place in - Yellow Ammonium sulfide - 20 drops
   Distilled water - 40 ml. for 1 minute.
8. Rinse in distilled water.

9. Counterstain with LT. GREEN (s) made into working solution - 15 seconds - 1 minute.
10. Wash well in 95% alcohol.
11. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid phosphatase - brown to black
Other elements - light green

Reference: Gomori, G: Stain Technology 5: 81, 1950.

ACID PHOSPHATASE - for Leukocytes

Fixation:

Formalin-Acetone for 1 minute at 0°C. 20% Formalin in 50% Acetone. (20 ml. Formaldehyde - 80 ml. of 50% Acetone)

Technique:

Air dried smears of peripheral blood or bone marrow. After fixation, air dry smears again, then rinse briefly in water. Use control slide (smear incubated in Stock Substrate Solution without Fast Blue BBN).

Prepare:

Working Substrate Solution

ACID PHOSPHATASE SUBSTRATE SOLUTION (s)..... 30 ml.

Fast Blue BBN..... 30 mg.

Dissolve and filter rapidly.

Staining Procedure:

1. Incubate fixed smears in a freshly prepared and filtered Working Substrate Solution at 37°C. for 1 hour.
2. Rinse in water and air dry.
3. Counterstain in 0.1% NEUTRAL RED SOLUTION (s) for 3 minutes. Air dry.
4. Dip in xylene and mount.

Results:

Acid phosphatase activity appears as round or rod-like dark-blue granules within the cytoplasm of the leukocytes, occasional red cell, and platelets. In some instances granules are superimposed on the nuclei. Nuclei are brilliant orange-red. Megakaryocytes are intensely reactive. All other myeloid elements of the bone marrow exhibit weak staining reaction. In peripheral blood mature and immature neutrophils and eosinophils exhibit weak to moderate activity. Monocytes generally weakly active although occasionally strongly reactive. Basophils cannot be differentiated from neutrophils.

Reference: Rosales, C.L., Bennett, J.M. and Rutenburg, A.M., British J. of Haematology, Vol. 12, No. 2, March 1966.

ALCOHOLIC HYALINE - Laqueur

Fixation:
10% Formalin or Zenker's

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Differentiating Solution
SATURATED ALCOHOLIC PICRIC ACID (s) ..... 10 ml.
20% Alcohol ..... 70 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain with MAYER'S HEMATOXYLIN (s) for 5 minutes.
3. Rinse in running water. Blue in tepid water.
4. Place slides on staining rack, pour filtered ANILINE ACID FUCHSIN(s) onto sections and heat with flame until solution is steaming. Let stain remain for 5 minutes.
5. Wash in running water for 5 minutes.
6. Differentiate with Differentiating Solution until only RBC and cytoplasmic hyaline bodies remain red.
7. Wash in running water for 10 minutes.
8. Mordant in 1% PHOSPHOMOLYBDIC ACID (s) for 4 hours.
9. Without washing, counterstain with LIGHT GREEN (s) for 1 hour.
10. Rinse in water.
11. Differentiate in 80% alcohol until collagen fibers remain green and background is gray-brown.
12. Dehydrate in alcohols, clear in xylene and mount.

Results:
Mallory bodies (cytoplasmic hyaline bodies) - red
Collagen - green
Nuclei - brown
Background - gray-brown

Reference: Laqueur, Am. J. Clin. Path., 20: 680, 1950.

ALKALINE PHOSPHATASE - Burstone

Fixation:

Cold Acetone

Technique:

Cut unfixed tissue on the Cryostat at 6 microns, mount on slides and allow to air dry before fixation. (Source of positive control - kidney, small intestine.)

Prepare:

Azo Dye Solution

5 mgs. of Napthol AS-MS, AS-AN, AS-CL, AS-TR, or AS-BI phosphate in 0.25 ml. of DMF, (dimethylformamide)

Add:

Distilled water..... 20 ml.

0.2 M TRIS BUFFER (pH 9.3) (s)..... 20 ml.

Red-Violet LB..... 30 mg.

Mix.

Filter into coplin jar and use immediately.

Staining Procedure:

1. Fix frozen sections in cold acetone briefly. (30 seconds).
2. Rinse in distilled water.
3. Place slides in Azo Dye Solution for 5-30 minutes. Check under microscope for reaction.
4. Wash in running water for 5 minutes.
5. Counterstain in MAYER'S HEMATOXYLIN(s) for 1 1/2 minutes.
6. Wash in running water for 10 minutes.
7. Mount in melted GLYCERIN-GELATIN (s).

Results:

Sites of alkaline phosphatase activity - red

Nuclei - blue

Reference: Bowling, M.C., *Histopathology Laboratory Procedures*, p. 46.

ALKALINE PHOSPHATASE - Gomori (Modified)

Fixation:

Cold 10% Formalin.

For Paraffin Blocks:

95% alcohol ..... 8 hours
100% alcohol ..... overnight
Chloroform I ..... 1 hour
Chloroform II ..... 1 hour
Chloroform 3 parts / Paraffin 1 part at 56° ..... 1 hour
Paraffin I ..... 1 hour
Paraffin II ..... 1 hour

Embed at not over 56°C. Cut at 4-6 microns.

Technique:

Cut 6-10 micron sections on the Cryostat (if unfixed). When spreading paraffin sections warming tray must not be over 37°C. Do not use egg albumin on slides. Source of positive control - kidney, small intestine.) Cut 6 slides. (3 for controls)

Prepare:

Alkaline Phosphatase Sodium Glycerophosphate Solution

ALKALINE PHOSPHATASE BUFFER SOLUTION (s) .... 4 parts
2% Sodium Beta Glycerophosphate (make fresh) ..... 1 part
For the Control Slides - make an equal amount of the above solution except substitute an equal amount of distilled water for the 2% Sodium Glycerophosphate.

Note: Solutions are kept refrigerated. Acid cleaned glassware must be used. If solutions turn milky, discard and start again with clean glassware and fresh solutions. Use a ventilating hood on Step 7 of the Staining Procedure. Avoid any contact with metal.

Staining Procedure:

1. Deparaffinize slides and hydrate to water. (Use fresh solutions).
2. Incubate 3 test slides at 37°C. in the Alkaline Phosphatase-Sodium Glycerophosphate Solution for 1-4 hours. Incubate 3 control slides in control solution for 2-4 hours.
3. Remove one test slide and one control slide at the following time - 1 hour - 2 1/2 hours - 4 hours.
4. Rinse quickly in several changes of distilled water.
5. Place in 2% COBALT NITRATE (s) for 3 minutes.
6. Rinse quickly in several changes of distilled water.
7. Place in Yellow Ammonium Sulfide ..... 10 drops
   Distilled water ..... 50 ml.
   for two minutes.
8. Wash thoroughly in tap water until the smell of ammonium sulfide is gone.
9. Counterstain with LT. GREEN (s) 15 seconds to 1 minute.
10. Wash well in 95% alcohol.
11. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Alkaline Phosphatase - black
Other elements - light green

Reference: Gomori, G., Proc. Soc. Exp. Biol. Med., 42: 23, 1939.

ALKALINE PHOSPHATASE - for Leukocytes

Fixation:

10% Formalin in absolute methanol at 0° - 4°C. for 30 seconds and rinsed quickly in water.

Technique:

Air dried blood smears. Use control slide. (Control slide is incubated in Stock Substrate Solution without Fast Blue BBN.)

Prepare:

Working Substrate Solution

ALKALINE PHOSPHATASE SUBSTRATE SOLUTION (s) 30 ml.
Fast Blue BBN..... 30 mg.
Dissolve and filter rapidly.

Staining Procedure:

1. Incubate fixed smears in a freshly prepared and filtered Working Substrate Solution at room temperature for 15 minutes.
2. Wash in 4 to 5 changes of water and air dry.
3. Counterstain in 0.1% NEUTRAL RED SOLUTION(s) for 3 minutes.
4. Rinse quickly in water and blot. Allow to dry.
5. Dip in xylene and mount.

Results:

Alkaline phosphatase activity is represented by discrete, bright blue granules of varying size predominantly in segmented neutrophils but also present in other cells of the myelocytic series except myeloblasts. Cytoplasmic granules of eosinophils do not stain. Basophils can not be differentiated from other granulocytes.
Nuclei - brilliant red-orange

Reference: Rutenburg, A.M., Rosales, C.L. and Bennett, J.M., J. Lab and Clin. Med., Vol. 65, No. 4, pp. 698-705, April 1965.

AMYLOID - Congo Red

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns. Use control slide.

Prepare:

Solution A
80% Alcohol saturated with Sodium Chloride
Solution B
1% Sodium Hydroxide
Solution C
80% Alcohol saturated with Congo Red and Sodium Chloride

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain for 2 minutes in MAYER'S HEMATOXYLIN (s).
3. Rinse in 3 changes of distilled water.
4. To 50 ml. of Solution A add 0.5 ml. of Solution B, filter and immerse slides for 20 minutes.*
5. To 50 ml. of Solution C, add 0.5 ml. of Solution B, filter and stain slides for 20 minutes.*
6. Dehydrate in 3 changes of absolute alcohol, clear in xylene and mount.
   The sections are to be examined both by direct transmitted illumination and by polarized light.
   *These solutions must be used within 15 minutes after the addition of sodium hydroxide.

Results:
In direct transmitted light, amyloid deposits will vary from orange to red in color. In addition to amyloid deposits, elastic tissue, cellulose fibers, the walls of a variety of fungi, and eosinophil granules will also stain orange to red. When viewed with polarized light, amyloid deposits will exhibit an intense green birefringence. A similar birefringence is also exhibited by cellulose derivatives and the walls of fungi. These latter materials can be distinguished from amyloid deposits by their characteristic morphology and by differential staining methods. Eosinophil granules and elastic tissue are not birefringent.

Reference: Puchtler et al. J. Histochem. Cytochem. 10, 355. 1962
See also:
1. DeLellis, R.A., Glenner, G.G., and Ram, J.S., Histochemical observations on amyloid with reference to polarization microscopy. J. Histochem. Cytochem. 16, 663, 1968.
2. DeLellis, R.A. and Bowling, M.C., The use of Sirus red and Congo red staining in routing histopathology. Human Path., 1, 655. 1970.

AMYLOID - Crystal Violet

Fixation:
10% Formalin

Technique:
Cut frozen or paraffin sections 10 microns. Use control slide.

Prepare:
Crystal Violet Solution
CRYSTAL VIOLET-AMYLOID (s) ..... 8 ml.
ACETIC ACID, 3% (s) ..... 32 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in Crystal Violet Solution for 5 minutes.
3. Wash in running water for 5 minutes.
4. Rinse in water.
5. Mount in water soluble mounting medium.

Note: Some tissues occasionally need to be differentiated in 1% ACETIC WATER (s) after Step 2. Slides should be seen by the investigator as soon as possible because the intensity of the stain fades.

Results:
Amyloid - red-purple
Background - blue-purple

Reference: Lillie, 3rd Ed. p. 518. Modification, M.C. Bowling, *Histopathology Laboratory Procedures,* Pathologic Anatomy Branch, NCI.

AMYLOID - Llewellyn's Progressive Sirius-Red

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 to 12 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) - 2 minutes. (It is not necessary to blue nuclei)
3. Rinse in distilled water, then in 70% alcohol.
4. Place in SIRIUS RED (LLEWELLYN'S) (s) for 1 hour.
5. Wash in tap water for 10 minutes.
6. Rinse quickly in 95% alcohol.
7. Dehydrate in 100% alcohol.
8. Clear in xylene and mount.

Results:
Amyloid - deep rose
Nuclei - blue
Eosinophil and paneth cell granules - deep rose.

Reference: Llewellyn, B.D.: J. Med. Lab. Technol., 27, pp. 308-309, 1970

AMYLOID - Slidder's Alcian Blue

Fixation:
10% Formalin

Technique:
Paraffin sections cut at 6 or 10 microns. Use control slide.

Prepare:
Acetic-Alcohol
95% Alcohol ..... 45 ml.
Distilled water ..... 45 ml.
Acetic acid ..... 10 ml.
Prepare fresh.
Alcian Blue Working Solution
ALCIAN BLUE ALCOHOLIC 1% (s) ..... 45 ml.
SODIUM SULFATE 1% (s) ..... 45 ml.
Acetic acid ..... 10 ml.
Let stand for 30 minutes.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Rinse briefly in distilled water.
3. Rinse in Acetic Alcohol.
4. Stain in Alcian Blue Working Solution for 2 hours.
5. Rinse in Acetic Alcohol.
6. Wash in distilled water 3 minutes.
7. Alkalinize for 30 minutes in SODIUM BORATE-SATURATED 80% ALCOHOLIC (s).
8. Wash in tap water for 3 minutes.
9. Rinse briefly in distilled water.
10. Stain nuclei with CELESTINE BLUE, LENDRUM'S (s) 3 to 5 minutes.
11. Rinse in distilled water.
12. Stain in MAYER'S HEMATOXYLIN (s) 1 1/2 minutes.
13. Wash in running tap water for 3 minutes.
14. Treat with PICRIC ACID-SATURATED ALCOHOLIC (s) for 1 minute.
15. Rinse in distilled water.
16. Stain in VAN GIESON (s) for 2 minutes.
17. *Do Not Wash;* dehydrate rapidly with absolute alcohol.
18. Clear in xylene and mount.

Results:
Amyloid (if recent) bright green; old amyloid paler green or blue, brown to red; nuclei black; connective tissue red; muscle, cytoplasm, erythrocytes and fresh fibrin yellow. Mast cell granules, certain colloids and various mucins stained by Alcian Blue react strongly. Aging fibrin sometimes takes a green shade; possibly at the same stage when it is sometimes metachromatic.

Reference: Private communication from W. Slidders.

AMYLOID - Sweat-Puchtler

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns. Use control slide.

Prepare:
1% Sodium Hydroxide
Alkaline Alcohol Solution
80% Alcohol ..... 99 ml.
1% Sodium Hydroxide ..... 1 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Mordant in BUFFERED NEUTRAL FORMALIN (s) overnight.
3. Wash in running water for 15 minutes.
4. Place in Alkaline Alcohol Solution for 1 hour.
5. Rinse in distilled water for 10 seconds.
6. Place in pre-heated SIRIUS RED SOLUTION (s) at 60°C. for 90 minutes.
7. Rinse quickly in 2 changes of 0.1 M SODIUM BORATE BUFFER pH 9.0 (s).
8. Wash in running water for 5 minutes.
9. Counterstain with MAYER'S HEMATOXYLIN (s) for 2 minutes.
10. Wash in running water for 5-10 minutes.
11. Drain and blot slides.
12. Dehydrate rapidly in absolute alcohols, clear in xylene and mount.

Results:
Amyloid - pink to red
Nuclei - blue
Elastica - pink to red

Reference: Sweat, F. and Puchtler, H.; Arch. Path. 80: 613-630, 1965.

ARGENTAFFIN CELLS - Methenamine Silver - Gomori-Burtner

Fixation:

10% Formalin

Technique:

Cut paraffin sections at 5-6 microns. Glassware must be chemically cleaned. Avoid metal.

Prepare:

Holmes Buffer pH 7.8

0.2 M BORIC ACID SOLUTION (s) ..... 16 ml.
.05 M SODIUM BORATE SOLUTION (s)..... 4 ml.

Working Silver Solution

METHENAMINE SILVER SOLUTION (s) ..... 30 ml.
Holmes Buffer pH 7.8 ..... 8 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Treat sections with WEIGERT'S IODINE SOLUTION (s) for 10 minutes.
3. Bleach with 5% SODIUM THIOSULFATE (s) for 2 minutes.
4. Wash in running water for 10 minutes.
5. Rinse in two changes of distilled water.
6. Place slides in Working Silver Solution at 60°C. for 1-3 hours. Check with microscope after 1 hour. (Impregnation time may be reduced by pre-heating silver solution.)
7. Rinse in distilled water.
8. Tone in 0.1% GOLD CHLORIDE (s) for 10 mintues.
9. Rinse in distilled water.
10. Fix in 5% SODIUM THIOSULFATE (s) for 2 minutes.
11. Wash in running water for 5 minutes.
12. Counterstain with SAFRANIN O (s) for 1/2 to 1 minute.
13. Dehydrate in alcohols, clear in xylene and mount.

Results:

Argentaffin cells - blackened
Coarse connective tissue of the submucose shows variable amount of blackening
Background - pink

Reference: Gomori, Burtner: Stain Technology, 24: 225, 1949.

ARGENTAFFIN (Carcinoid Tumors) - Sevier-Munger - Modification

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 5 microns.

Prepare:
Ammoniacal Silver Working Solution

a) Into a 200 ml. beaker, place 50 ml. of 10% SILVER NITRATE (s).
b) Add Ammonium Hydroxide (28%) (small fresh bottles stored in refrigerator) drop by drop until the dark brown precipitate which forms has almost disappeared. Avoid complete decolorization. Swirl vigorously between drops.
c) Add to this solution 0.5 ml. of SODIUM CARBONATE SOLUTION (s). Mix well.
d) Add 25 drops of cold, fresh Ammonium Hydroxide (28%). Swirl vigorously. Solution should now be clear. Cover.
e) Just before use - add 10 drops of 2% FORMALIN (s). Swirl.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place coplin jar of 20% SILVER NITRATE (s) in 60°C. oven. Allow 30 minutes for solution to warm.
3. Blot water from slides and place on a 40°C. warming tray until slides are warm and dry.
4. Place slides in warm silver solution and return to 60°C. oven for 10-40 minutes or until tissue is a light tan color.
5. Take slides from oven, wash in 2 changes of tap water, drain and place into clean, dry coplin jar. Pour Ammoniacal Silver Solution over slides.
6. Develop until sections are golden brown 5-30 minutes. At this stage slides must be checked under the microscope until proper development has been reached. Nerve fibers should be black, nuclei light gray or black, background - golden brown.
7. Wash slides well in several changes of distilled water.
8. Place in 5% SODIUM THIOSULFATE (s) for 2 minutes.
9. Wash in running water for 2 minutes.
10. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nerve fibers - black
Red cells - dark brown
Basement membrane - brown
Nuclei - light gray or black
Argentaffin granules - black
Background - golden yellow
Argyrophil granules -black

Notes:

1. Glassware for silver must be acid cleaned.
2. It is suggested that duplicate slides are run simultaneously, one of which is developed light and one left longer in the ammoniacal silver and developed darker.
3. Check oven temperature to be sure it is 60°C.
4. The 20% SILVER NITRATE (s) can be reused numerous times. Let cool and filter each time before returning it to the refrigerator for storage.
5. 5% SODIUM BICARBONATE (s) may be used in place of SODIUM CARBONATE SOLUTION (s) with equal results.
6. Precipitation is lessened in the Ammoniacal Silver Solution when 5% SODIUM BICARBONATE (s) is used.

Reference: A.C. Sevier and B.L. Munger, A Silver Method for Paraffin Section of Neural Tissue., J. Neuropath. Exp. Neuro. 24: 130-135, 1965.

AZZOPARDI - Modification of the Bodian Argyrophil Technique

Fixation:

10% Formalin or Bouin's

Technique:

Cut paraffin sections at 5 microns. If tissue flakes from the slides, the use of gelatinized slides is suggested. See stock index for gelatin solution. Glassware should be acid cleaned.

Prepare:

1% Protargol Solution

a) Clean 5-6 grams of copper shot in 1% Hydrochloric acid for 10 minutes. This will prevent tissue from becoming impregnated with silver, which tends to obliterate cellular detail. Wash well in running water. Rinse in several changes of distilled water. Drain and place shot evenly in the bottom of a coplin jar.

b) Gently sprinkle 1 gram of protargol on the surface of 100 ml. of double distilled water without heat and place in 60°C. oven. Allow to remain undisturbed until dissolved (10 minutes). Pour this solution over the cleaned copper shot.

Reducing Solution (make fresh)

Hydroquinone	1 gm.
Sodium Bisulfite $NaHSO_3$	5 gm.
Distilled water	100 ml.

2% Oxalic Acid Solution (make fresh)

Oxalic Acid	2 gm.
Distilled water	100 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Wash well in several changes of double distilled water.
3. Place slides in Protargol Solution. Leave at 37°C. for 48 hours.
4. Remove excess silver deposit from back and ends of slides and wash well in double distilled water.*
5. Place slides in Reducing Solution for 10 minutes.
6. Wash well in double distilled water.
7. Tone in GOLD CHLORIDE (s) - 5 minutes.
8. Wash well in distilled water.
9. Place slides in 2% Oxalic Acid Solution until purple tinge develops - 2-4 minutes.
10. Wash well in distilled water.
11. Place slides in 5% SODIUM THIOSULFATE SOLUTION (s) for 2 minutes.
12. Wash in running tap water for 2 minutes.
13. May be counterstained with VAN GIESON (s) 1-3 minutes.
14. Rinse in distilled water.
15. Dehydrate in alcohols, clear in xylene and mount.

Results:

Argentaffin Cells - black
Argyrophilic granules - black
Nerve fibers - black
Background - pale gray blue - if counterstained - yellow-green
Nuclei - black

Note: This is actually the Bodian Silver Technique. The only modification is the addition of glacial acetic acid in the gold toning solution. We list it under "Azzopardi" because of the interest of our investigators in carcinoid and its relation to the paper cited in the reference.

We thank Dr. DeLellis for the following definitions.

Argyrophil - A substance which has the ability to become impregnated with already reduced silver.

Argentaffin - A substance which has the ability to directly reduce silver.

*If sections tend to loosen from slide, place them briefly in an acid rinse. 0.5 ml. of 2% acetic acid in 50 ml. distilled water.

Reference: Azzopardi, J.G., and Pollock, D.J.: Argentaffin Argyrophil Cells in Gastric Carcinoma, J. of Path. and Bacteriology, Vol. 86, No. 2, 443-451, 1963.

BACTERIA - Brown and Brenn (Modification)

Fixation:
Formalin preferred.

Technique:
Paraffin sections cut at 4 to 6 microns. Use control slide.

Prepare:
Working Crystal Violet Solution
1% CRYSTAL VIOLET (s) ..... 4 parts
5% SODIUM BICARBONATE SOLUTION (s) ..... 1 part
Working Basic Fuchsin Solution
BASIC FUSHSIN SOLUTION, SAT. AQ. (s) ..... 20 drops
Distilled water ..... 50 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water. Stain control slide first.
2. Stain in Crystal Violet Working Solution for 2 minutes.
3. Wash in running tap water.
4. Place in WEIGERT'S IODINE (s) for 30 seconds.
5. Rinse in running tap water and blot with filter paper to remove excess water. (blot only if easily decolorized.)
6. Decolorize in acetone until blue color no longer runs off slide. (This step must be done with care and usually takes just a few seconds.)
7. Stain with Working Basic Fuchsin Solution for 5 minutes.
8. Wash in running tap water, blot gently but not completely dry.
9. Dip quickly in acetone to start reaction.
10. Differentiate immediately with PICRIC ACID-ACETONE SOLUTION (s) until sections are yellowish-pink.
11. Rinse quickly in acetone and in a mixture of equal parts of acetone and xylene.
12. Clear in several changes of xylene and mount.

Results:
Gram-positive bacteria - blue
Gram-negative bacteria - red
Nuclei - red
Other tissue elements - yellow
If nuclei are blue the sections are not properly decolorized.

Reference: Brown, J.H., and Brenn, L.; Bulletin of the Johns Hopkins Hopsital, 48: 69, 1931. (NCI Modification, Path. Tech. Section)

BACTERIA-FUNGI - Cresyl Violet Acetate

Fixation:
Any

Technique:
Cut paraffin sections at 5 microns. Use control slide.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in CRESYL VIOLET ACETATE (s) - 5 minutes.
3. Wash in running tap water - 5 minutes.
4. Counterstain with METANIL YELLOW (s) or LT. GREEN (s) if desired, with excellent results - 1 minute.
5. Drain slides well, dehydrate rapidly in 3 changes of absolute alcohol.
6. Clear in 2 changes of xylene and mount.

Results:
Bacteria and fungi - blue to purple
Nissl substance and lipofucsin - dark blue
Cartilage and mucus - pink to red
Background - pale blue without counterstain
with counterstain - pale yellow or light green

Reference: Puchtler H. and Sweat F., Stain Technology, Vol. 39, No. 1, pg. 1, Jan. 1964.

BACTERIA - Humberstone (modified)

Fixation:
10% Formalin preferred.

Technique:
Cut paraffin sections at 6 microns. Use control slide.

Prepare:
Basic Fuchsin Working Solution
BASIC FUCHSIN - SATURATED (s)..... 10 drops
Distilled water..... 50 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place in 1% CRYSTAL VIOLET (s) for 1 minute.
3. Wash in tap water.
4. Place in GRAM'S IODINE (s) for 1 minute.
5. Wash in distilled water.
6. Decolorize with acetone until clear.
7. Immediately wash well in distilled water.
8. Place in Basic Fuchsin Working Solution for 1/2 to 1 minute.
9. Wash in distilled water.
10. Place in GALLEGO'S SOLUTION (s) 2 changes - 1 minute each.
11. Wash in distilled water.
12. Rinse in acetone for 30 seconds.
13. Place in ACETONE-PICRIC ACID (s) for 2 minutes.
14. Dehydrate in 2 changes 1/2 acetone - 1/2 xylene.
15. Clear in several changes of xylene and mount.

Results:
Gram-positive bacteria - blue
Gram-negative bacteria - red
Nuclei - red
If nuclei are blue the sections are not properly decolorized.

Reference: Communication with AFIP.

BACTERIA - MacCallum-Goodpasture

Fixation:

Any well-fixed tissue. Zenker's or Orth's preferred.

Technique:

Cut paraffin sections at 6 microns. Use control slide.

Prepare:

Stirling's Gentian Violet Solution

Gentian Violet (crystal violet)	5 gm.
Alcohol, absolute	10 ml.
Aniline*(do not pipette)	2 ml.
Distilled water	88 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain with GOODPASTURE'S SOLUTION (s) for 10 minutes.
3. Rinse in distilled water.
4. Differentiate in Formaldehyde (37-40%) for a few minutes. This also fixes the stain.
5. Wash in running water for 3 minutes.
6. Place in PICRIC ACID-SATURATED AQUEOUS (s) for 3-5 minutes.
7. Rinse in distilled water.
8. Differentiate in 95% alcohol for 30 seconds.
9. Rinse in distilled water.
10. Stain in Stirling's Gentian Violet Solution for 3 minutes.
11. Rinse in distilled water.
12. Place in GRAM'S IODINE (s) for 1 minute.
13. Rinse in distilled water. Blot - not to complete dryness.
14. Differentiate in equal parts of aniline and xylene - several changes - until sections appear light purplish-red.
15. Clear in xylene and mount.

Results:

Gram-positive organisms - blue
Gram-negative organisms - red
Other elements - shade of red to purple

Caution:

*Aniline is hazardous to use and should be handled with extreme care. It should not be ingested, inhaled or absorbed through the skin. Never pipette.

Reference: MacCallum, W.G., JAMA 72: 193, 1919.

NOTES ON GRAM STAIN

1. The concentration of the Crystal Violet (up to 2%) and the concentration of iodine (up to 1%) gives the allowable range of the decolorization time to be broadened and the result is more dependable. *Decolorization* is slowed.
   Crystal Violet ..... 2 minutes.
2. The iodine step makes cells easier to decolorize although it retards the overstaining of the counterstain. Iodine step must not be cut down. Iodine.....30 seconds; Weigerts.....1%.
3. If counterstaining is prolonged the crystal violet (Gram-positive bacteria) may be replaced by the counter-stain.
4. Decolorizers

   95% ethyl alcohol..... 15 seconds - 3 minutes.
   Acetone..... 5 seconds - 2 minutes.
   n-propyl alcohol ..... 30 seconds - 30 minutes.

   Acetone is quicker but the reliability is increased with 95% alcohol or n-propyl alcohol.

   n-propyl decolorization of 6 minutes is reliable with a safety factor of 3 minutes.

   Dilution with water (up to 20% of acetone and 95% alcohol) speeds decolorization.

   90% n-propyl however is still safer.

   Wet slides decolorize more rapidly than dry slides.

   If iodine is permitted to accumulate in the decolorizer - the rate of decolorization slows.

Reference: *Selective Histochemical and Histopathological Methods* by Thompson, pg. 1005.

BASEMENT MEMBRANE - Periodic Acid Methenamine Silver (PAMS)

Fixation:
Any

Technique:
Cut paraffin sections 3 to 5 microns. Use acid clean glassware.

Prepare:
3% Methenamine
Gomori's Silver Methenamine
3% Methenamine ..... 100 ml.
Add:
5% SILVER NITRATE (s) ..... 5 ml.
Add:
5% BORAX (s) ..... 12 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. PERIODIC ACID 0.5% SOLUTION (s) at room temperature for 15-20 minutes.
3. Wash well in distilled water.
4. Place in Working Silver Methenamine Solution for 2-4 hours at 45-60°C. Change the solution every 2 hours. Progress of staining should be checked under the microscope. Rinse the slide in warm distilled water and examine; if basement membrane is too light, return to staining solution.
5. Wash in distilled water.
6. Tone in 0.2% GOLD CHLORIDE (s) for 1 to 2 minutes.
7. Wash in distilled water.
8. Place in 3% SODIUM THIOSULFATE (s) for 3 minutes.
9. Wash well in running water for 5 minutes.
10. If desired, counterstain with hematoxylin and eosin or trichrome stain.
11. Dehydrate, clear and mount.

Results:
Basement membrane - black
Reticulum fibers - black
Nuclei - blue
Cytoplasm, collagen and connective tissue - pink to orange.

Reference: Jones, Am. J. Path., 33: 313, 1957.

BILE - Hall

Fixation:
Formalin

Technique:
Paraffin sections cut at 6 microns or frozen sections. Use control slide.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain 5 minutes in freshly filtered FOUCHET's REAGENT (s).
3. Wash in tap water, then distilled water.
4. Stain in VAN GIESON (s) for 5 minutes.
5. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Bilirubin oxidized to Biliverdin - olive drab green to emerald green, depending upon the concentration of bilirubin.

Reference: Hall, M.J.: Amer. J. of Clin. Path., 35: 313-316, 1960.

BILE CANALICULI - Modification

Fixation:

10% Formalin is preferred.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Oxalic Acid, 1%

Acid Fuchsin Working Solution

ACID FUCHSIN, 1% (s)	10.0 ml.
Oxalic Acid, 1%	1.0 ml.
Distilled water	80.0 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Rinse in distilled water.
3. Place in CELESTINE BLUE (s) for 30 minutes.
4. Rinse in distilled water.
5. Place in 1% PHOSPHOTUNGSTIC ACID (s) for 15 minutes.
6. Wash in tap water for 3 minutes.
7. Place in Acid Fuchsin Working Solution for 8 minutes.
8. Dehydrate quickly in alcohols, clear in xylene and mount.

Results:

Bile canaliculi - pink to red

Nuclei - blue

Reference: Luna, L., Ishak, K., Amer. J. of Med. Tech., Vol. 33, No. 6, 1967.

BILE CANALICULI - Regaud's

Fixation:
Zenker-Formol

Technique:
Cut paraffin sections at 5 microns

Prepare:
5% Iron Alum (ferric Ammonium Sulfate)
2.5% Iron Alum

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Mordant in 5% Iron Alum for 2 hours.
3. Wash briefly in distilled water.
4. Stain in REGAUD'S HEMATOXYLIN (s) for 2 hours.
5. Wash in water.
6. Decolorize in 5% Iron Alum for a short time and place in 2.5% Iron Alum for detailed differentiation, controlling the result with the microscope.
7. Wash in running water for 15-30 minutes.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:
Bile capillaries - blue
Nuclei - blue

BILE CAPILLARIES - Clara

Fixation:

10% Alcoholic Formalin preferred. Others may be used.

Technique:

Cut paraffin sections at 4-6 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in CLARA'S HEMATOXYLIN SOLUTION A (s) for 24 hours at 50°C.
3. Rinse in distilled water.
4. Stain in CLARA'S HEMATOXYLIN SOLUTION B (s) until bile capillaries are stained - 15-30 minutes.
5. Wash briefly in running water. Let slides blue.
6. Rinse in distilled water.
7. Dehydrate in alcohols, clear in xylene and mount.

Results:

Bile capillaries - blue

Reference: Gray, Peter, *The Microtomist's Formulary and Guide,* pp. 422, 1954.

BILE PIGMENT - Stein, Modification

Fixation:
Alcohol or 10% Formalin

Technique:
Cut paraffin sections at 6-7 microns. Use control slide.

Prepare:
Stein's Working Iodine Solution - Must be fresh.
TINCTURE OF IODINE (s) ..... 10 ml.
LUGOL'S IODINE (s) ..... 30 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place in Stein's Working Iodine Solution for 6-12 hours.
3. Wash in distilled water.
4. Decolorize in 5% SODIUM THIOSULFATE (s) for 15 - 30 seconds. Wash in tap water 2-3 minutes.
5. Wash in distilled water.
6. Counterstain with NUCLEAR FAST RED (Kernechtrot) (s) for 5 minutes.
7. Rinse in distilled water.
8. Dehydrate quickly in acetone, clear in xylene and mount.

Results:
Bile pigment - emerald green
Nuclei - red
Cytoplasm - pink to rose

Reference: Glick, D.; *Techniques of Histochemistry and Cytochemistry,* pg. 63, 1949.

CALCIUM - Alizarin Red S - Langeron

Fixation:
Any

Technique:
Paraffin sections cut at 6 microns. Use control slide.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain 1 hour in ALIZARIN RED S (s).
3. Rinse in water.
4. Dehydrate in *acetone,* clear and mount in xylene. (May be counterstained with METHYLENE BLUE (s).

Results:
Calcium deposits - scarlet
Cartilage - purple to violet
Nuclei and other tissue - blue (if counterstain is used)

Reference: Lillie, R.D.: *Histopathologic Technic,* The Blakiston Company, Philadelphia, p. 249, 1948, 1st. Ed.

CALCIUM - Von Kossa

Fixation:

Alcohol preferred. 10% Formalin may be used.

Technique:

Cut paraffin sections at 6 microns. Use control slide. Glassware must be acid cleaned.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in 5% SILVER NITRATE (s) for 30-60 minutes, exposed to direct sunlight, ultra-violet or 100 watt desk lamp.
3. Rinse in distilled water.
4. Place in 5% SODIUM THIOSULFATE (s) for 2-3 minutes.
5. Wash in tap water 2 minutes, then wash well in distilled water.
6. Counterstain with NUCLEAR FAST RED (Kernechtrot) (s) for 5 minutes.
7. Wash well in distilled water.
8. Dehydrate in alcohol, clear in xylene and mount.

Results:

Calcium salts - black
Nuclei - red
Cytoplasm - pink to rose

Reference: Mallory, F.B., *Pathological Technique,* p. 144, 1968.

CARBOHYDRATE SUBSTANCES

POLYSACCHARIDES

GLYCOGEN is the chief carbohydrate storage material in animals. It is formed by and mostly stored in the liver. It is changed to glucose and liberated as needed. Glycogen is the only polysaccharide found in higher animals.

GLYCOGEN IS:

1. PAS Positive.
2. PAS Positive after lipid extraction.
3. Not metachromatic.
4. Weakly metachromatic after sulfation or chromic acid oxidation.
5. Negative with Alcian Blue, Astra Blue and Mowry's Colloidal Iron.
6. Can be digested with Diastase.

MUCOPOLYSACCHARIDES

CONTAIN HEXOSAMINE

A. *ACID MUCOPOLYSACCHARIDES*
(usually PAS Negative)

1. Hyaluronic Acid - found in ocular fluid, umbilical cord, skin, cornea, synovial fluid, mesothelioma, hemolytic strep and tendons.
2. Hyaluronsulfate - found in cornea.
3. Chondroitin sulfates - found in cornea. They confer metachromasia.
4. Heparin - a sulfated mucopolysaccharide produced by mast cells which also produce hyaluronic acid. Heparin is strongly metachromic. Mast cells may be PAS positive or negative.
5. Mucoitin sulfates. Found in mucin.
6. Keratosulfate - resistant to hyaluronidase.
7. Limacoitinsulfate - found in snail mucin.
8. Sialomucins - vary markedly in rate of hydrolysis from sites within a species and between species. Sialomucins can be eliminated with sialidase (neuraminidase) Sources: a.) Vibrio cholerae (best) b.) Clostridium perfringens c.) influenza vaccines.

B. *NEUTRAL MUCOPOLYSACCHARIDES*
(PAS Positive)
Found in pig gastric mucosa, chitin, and fungi. They are firmly bound to protein and histochemically similar to mucoproteins.
TO DIFFERENTIATE A AND B

A.1. Positive with -
   a. Alcian Blue pH 2.4 - 2.6.
   b. Astra Blue pH 2 (Pioch)

c. Hal's Modification of Mowry's Colloidal Iron. All may be combined with PAS
2. Usually PAS negative.
3. Eliminated by sulfation.
4. Sulfated acid mucopolysaccharides are metachromatic. Metachromasia can be eliminated by sulfation.

B.1. Positive with -
a. PAS
b. PAS after lipid extraction.
c. Metachromic dyes such as Toluidine Blue, Thionin, and Azure A.

MUCOPROTEINS

CONTAIN HEXOSAMINE
Proteins (Peptides) are the major component of mucoproteins.
Commonly investigated mucoproteins are:
1. Blood group aggulatinogens.
2. Mucosubstances of the cervix.
3. Digestive tract
4. Respiratory tract
5. Gonadotropic hormones.
6. Thyrotropic hormones
7. Thyroglobulin
8. Hen eggs
9. Mucoid of frog oviduct.

Mucoproteins can be differentiated from Polysaccharides with diastase digestion followed with PAS. Mucoproteins can be differentiated from lipids by lipid extraction (acetylation) followed with PAS.

GLYCOPROTEINS

CONTAINS LESS THAN 4% HEXOSAMINE
Glycoproteins are found in:
1. Ovalbumin
2. Serum Albumin
3. Collagen
4. Reticulum

GLYCOLIPIDS

(Lipids containing carbohydrates)
The best known representatives of this group are cerebrosides

LIPIDS

A. Glycolipids
   1. Cerebrosides
   2. Gangliosides (kerasin in Gaucher cells)

B. Phospholipids
   1. Sphingomyelin
   2. Cardiolipid
   3. Brain Diphosphoinositide
   4. Cephalin

C. Pigments of lipid nature.
   1. Ceroid
   2. Melanosis coli pigment
   3. Adrenal fuscin
   4. Some lipofuscin

GLYCOLIPIDS ARE PAS positive.
Lipids can be extracted by acetylation.

MUCOPROTEIN AND GLYCOPROTEIN REACTIONS ARE IDENTICAL AND CANNOT BE DIFFERENTIATED BOTH ARE:

1. PAS Positive
2. PAS Positive after lipid extraction
3. Alcian Blue negative
4. Astra Blue negative
5. Colloidal Iron negative
6. Diastase fast
7. Hyaluronidase fast
8. Not Metachromatic
9. Weakly metachromatic sulfation or chromic acid extraction
10. Protein reaction variable

Reference: *Histochemistry, Theory, Practice and Bibliography,* Chapter, III., Barka and Anderson, 1963.

CARBOHYDRATES - continued

Histochemical Identification of Carbohydrate Substances

With the exception of glycogen, carbohydrates can be classified but not specifically identified by present histochemical techniques. Neutral mucopolysaccharides cannot be distinguished from mucoproteins, although the former do not occur in significant amounts in higher animals. Similarly, no histochemical distinction can be drawn between glycoproteins and mucoproteins.
The following scheme may assist in the identification of carbohydrate substances.

POSITIVE	NEGATIVE
PAS Reaction	
Neutral mucopolysaccharides Mucoproteins Glycoproteins Glycolipids Sphingolipids Proteins (very weak)	Acid mucopolysaccharides
PAS Reaction After Lipid Extraction	
Glycogen Neutral mucopolysaccharides Mucoproteins Glycoproteins Proteins (very weak)	Acid mucopolysaccharides
Metachromasy	
Acid mucopolysaccharides Hyaluronic acid: weak Sulfate esters: strong, alcohol resistant Sphingolipids (if preserved)	Glycogen Neutral mucopolysaccharides Mucoproteins Glycoproteins
Metachromasy after Sulfation or Chromic Acid Oxidation	
Acid mucopolysaccharides Sphingolipids Glycogen (weak) Neutral mucopolysaccharides (weak) Mucoproteins (weak) Glycoproteins (weak)	
Alcian Blue, Astra Blue Stainings, Hale Reaction	
Acid mucopolysaccharides	Neutral mucopolysaccharides Mucoproteins Glycoproteins Glycogen

POSITIVE	NEGATIVE
Methylene Blue Extinction	
Below pH 4	*Above pH 4*
Acid mucopolysaccharides	Neutral mucopolysaccharides
Glycolipids (1)	Mucoproteins
	Glycoproteins

Enzyme Digestion

Diastase: Glycogen labile, others fast.
Hyalurondiase (Testicular): Chondroitin sulfate A and C labile, others fast.

Protein Reaction

Glycogen is generally not associated with proteins in the stainings. Other carbohydrate-containing structures give variable protein stainings.

Reference: Barka T. and Anderson, P.J., *Histochemistry,* pp. 68-69, 1963.

CARBOHYDRATES

Acetylation

This method may be used to distinguish lipid from non-lipid PAS-positive substances. Acetylation blocks both the hydroxyl and amino groups thereby preventing oxidation by periodic acid.

Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in a mixture of —
   Acetic anhydride ..... 2 parts
   Pyridine ..... 3 parts
   for 1-24 hours at room temperature or 1/2 - 6 hours at 58°C.
3. Rinse in alcohol for 1-2 minutes.
4. Rinse briefly in distilled water.
5. Stain as usual with PAS. Stain a control slide without acetylation.

Results:

The staining of lipid components may be diminished since pyridine may act as a lipid solvent.

Sulfation

Sulfation eliminates acid mucopolysaccharides.

Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in a solution of equal parts of concentrated sulfuric acid and glacial acetic acid at 0°C. for 10 minutes.
   Note: Mix with caution. Wear gloves. Place container in ice bath.
3. Wash in several changes of glacial acetic acid for 2-3 minutes.
4. Wash in running water. Rinse in distilled water.
5. Stain with a metachromatic stain. Stain a control slide without sulfation.

Results:

Induced metachromasia indicates the presence of neutral mucopolysaccharides or mucoproteins.

CARBOHYDRATES

Methylation

This method destroys the staining reaction of acid polysaccharides by Alcian Blue, Muller-Mowry colloidal iron and Toluidine Blue metachromasia.

Procedure:

1. Deparaffinize duplicate slides.
2. Pass through two changes of absolute alcohol and two changes of methyl alcohol.
3. (a) Treat test slide in—
   Absolute methyl alcohol ..... 40 ml.
   Concentrated hydrochloric acid ..... 0.3 ml.
   at 58°C. for 3 hours.
   (b) Treat duplicate control slide in methyl alcohol at 58°C. for 3 hours. To prevent evaporation, seal the coplin jars by greasing the contact surface of the coplin jar lids with vaseline.
4. Rinse both slides in 70% alcohol and wash in tap water.
5. Perform staining procedure of choice.

Results:

Staining of acidic carbohydrates should be unaffected in the control slide but abolished in the test slide.

Methylene Blue Extinction Method

Fixation:

10% Formalin, Carnoy's, Zenker's or Zenker-Formol.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Veronal-Acetate Buffer - Michaelis

Sodium Acetate ($CH_3COONa.3H_2O$) ..... 9.714 gm.
Sodium Barbituate ..... 14.714 gm.
Make up to 500 ml. with distilled water.

0.64% Methylene Blue Solution.

Dilution Series

#1 Coplin jar - pH 2.62
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 16 ml.
Distilled water ..... 4 ml.

#2 Coplin jar - pH 3.62
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 14 ml.
Distilled water ..... 6 ml.

#3 Coplin jar - pH 4.66
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 10 ml.
Distilled water ..... 10 ml.

#4 Coplin jar - pH 5.32
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 8 ml.
Distilled water ..... 12 ml.

#5 Coplin jar - pH 6.12
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 7 ml.
Distilled water ..... 13 ml.

#6 Coplin jar - pH 6.75
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 6.5 ml.
Distilled water ..... 13.5 ml.

#7 Coplin jar - pH 7.66
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 4 ml.
Distilled water ..... 16 ml.

#8 Coplin jar - pH 7.90
Buffer solution ..... 5 ml.
0.1N HCl (s) ..... 3 ml.
Distilled water ..... 17 ml.

#9 Coplin jar - pH 8.18
Buffer solution ..... 5 ml.

0.1N HCl (s) ..... 2 ml.
Distilled water ..... 18 ml.
To each Coplin jar add 0.5 ml. of 0.64% Methylene Blue Solution.

Staining Procedure:

1. Stain parallel sections in each of the 9 Methylene Blue Solutions for 20-24 hours.
2. Rinse in distilled water and mount in melted glycerine jelly. Microscopic evaluation should be made immediately.

Results:

This is used as an auxiliary method to differentiate acid mucopolysaccharides from mucoproteins and other proteins. Structures staining below pH 3 (and the staining is not due to nucleic acids) the presence of acid mucopolysaccharides is indicated. Sulfated mucopolysaccharides (heparin in mast cells) are stained below pH 2.

Reference: Barka, T., Anderson, P.: *Histochemistry,* pp. 81-83, 1963.

Classification of Mucosubstances

This classification is closely based on that of Spicer et al. (1965) and represents the latest classification scheme.

I. Neutral Mucosubstances

Neutral glycoproteins
Immunoglobulins
Fuco-mucoids (All PAS positive Periodate reactive)
Manno-mucoids
Found: Gastric surface epithelia, Thyroid colloid, (Man Guinea Pig, Rabbit) Coagulating Gland Fluid, (Rat, Mouse).

II. Acid Mucosubstances

A. Sulfated
(1) Connective Tissue Mucopolysaccharides
(Pas negative Periodate unreactive)
(a) Stable to testicular hyaluronidase
(i) Alcianophilic in 1.0 M Magnesium Chlorid
(Keratan sulfate, heparin)
Found: Cornea and Mast Cells
(ii) Alcianophilic in 0.7 M Magnesium Chloride
(Dermatan sulfate)
(b) Labile to testicular hyaluronidase
(i) Alcohol resistant azurophilia (0.02% Azure A at or above pH 2.0)
(Chondroitin sulfates in cartilage)
Found: Ovarian Follicle Fluid, Cartilage
(ii) Alcohol resistant azurophilia (0.02% Azure A at or above pH 4.0)

(Chondroitin sulfates in vascular walls)
Found: Aorta, Heart valves, Renal papilla, some areas of cartilage.
(2) Epithelial Sulfomucins (testicular hyaluronidase resistant)
(a) PAS negative
(i) Sulfate esters on vic-glycols
Found: Sublingual glands, (Hamster) Glossal mucus glands, (Rabbit) Colonic Goblets, (Rabbit).
(ii) Sulfate esters not on vic-glycols
(a) Alcohol resistant Azurophilia (0.02% Azure A at or above pH 2.0).
Found: Colon (Guinea pig)
(b) Alcohol resistant Azurophilia (0.02% Azure A at or above pH 4.5)
Found: Duodenal Goblets, (Rat, Mouse) Pyloric Glands, (Rat, Mouse)

B. Non-Sulfated
(1) Hexuronic acid-rich mucopolysaccharides (Hyaluronic Acid)
Found: Ganglion Cysts of Synovia, (Man) Vitreous, (Man), Cock's Comb, Estrogen treated cervix uteri (Mouse)
(2) Sialic acid-rich mucosubstances
(a) Connective tissue sialomucins
Found: Cartilage
(b) Epithelial sialomucins
(i) Labile to Neuraminidase, PAS positive, metachromatic with Azure A.
Found: Sublingual glands (Mouse, Hamster, Guinea Pig)
(ii) Slowly digested by Neuraminidase
(a) PAS positive
Found: Vaginal epithelium (Pregnant Mouse)
(b) PAS negative
Found: Rectosigmoid mucus cells (Mouse)
(iii) Stable to Neuraminidase
(a) Rendered metachromatic and susceptible to enzyme by prior saponification.
Found: Sublingual Gland (Rat)
(b) Stable to Neuraminidase after saponification.
(i) PAS positive
Found: Sublingual Gland (Man, Monkey)
(ii) PAS negative
Found: Mammary Gland secretion (Mouse)

Reference: Pearse, *Histochemistry, Theoretical and Applied,* Vol. 1, pp. 305-307, 3rd. Ed. 1968.

CHOLESTEROL - Schultz

Fixation:

10% Formalin. Do not transfer to alcohol.

Technique:

Frozen sections cut at 10 microns.

Prepare:

2.5% Ferric Ammonium Sulfate

Glacial Acetic-Sulfuric Acid Solution

Prepare fresh before using.

Acetic acid ..... 5 ml.

Sulfuric acid, concentrated ..... 5 ml.

Add the sulfuric acid slowly, constantly stirring, to the acetic acid in a flask cooled in an ice bath. Wear safety goggles and acid resistant gloves.

Staining Procedure:

1. Collect frozen section in distilled water.
2. Place sections in the Ferric Ammonium Sulfate Solution for 3 days.
3. Rinse in 3 changes of distilled water.
4. Float sections onto slides and blot dry. Slides may be held at this point until investigator is ready for wet reading.
5. Place one drop of freshly prepared Glacial Acetic-Sulfuric Acid Solution on the section.
6. Apply coverslip immediately.
7. Examine microscopically within a few minutes.

Results:

Cholesterol - green, blue or blue-green

Reference: Schultz, A., Zbl. Allg. Path., 35: 314, 1924.

CHLORACETATE ESTERASE AND NON-SPECIFIC ESTERASES

Fixation:

Buffered formalin acetone:

Sodium Phosphate (Na_2HPO_4)	20 mg.
Potassium Phosphate (KH_2PO_4)	100 mg.
Water, tap (H_2O)	30 ml.
Acetone	45 ml.
Formalin	25 ml.

Adjust to pH 6.6 and refrigerate.

Technique:

Air dried smears fixed in Buffered formalin acetone for 30 seconds at 4-10°C. Wash in 3 changes of distilled water then air dry at room temperature for 10 - 30 minutes.

USE ACID CLEAN GLASSWARE OR NEW DISPOSABLE BEAKERS.

Prepare:

Sorenson's Buffer (pH 7.6) ..... 100 ml.

Check with pH meter.

Solution A

Sorenson's Buffer (pH 7.6) ..... 26.7 ml.

Solution B

Pararosanilin hydrochloride	1 gm.
Distilled water	20 ml.
Hydrochloric acid	5 ml.

Dissolve by gentle warming. Filter after cooling. Stable for several days.

Solution C

Sodium Nitrite	0.4 gm.
Distilled water	10 ml.

MUST BE MADE FRESH.

Solution D

*Alpha Naphthyl Acetate	30 mg.
Ethylene glycol monomethyl ether	1.5 ml.

Solution E

Sorenson's Buffer (pH 7.4) ..... 28.5 ml.

(Use remaining original buffer and change pH)

Check with pH meter.

Solution F - MAKE IMMEDIATELY BEFORE USE.

*Napthol AS-D Chloroacetate	3 mg.
N-N dimethyl formamide	1.5 ml.

Solution G

**Fast Blue BBN	15 mg.

Set aside in beaker till ready for use.

1% Methyl Green

Staining Procedure:

1. Mix 0.9 ml. *Solution B* with 0.9 ml. *Solution C.* Let stand for 1 minute.
2. Add *Solution A* and mix well.
3. To *Solution D,* add solutions from step 2. Adjust pH to 6.1. Filter. Immediately place slides in this solution for 45 minutes.
4. Wash in running water for 5 minutes.
5. Rinse in 3 changes of distilled water.
6. Make *Solution F.* Immediately combine with *Solution E,* mixing well. Pour this solution into beaker containing *Solution G.* Mix well and filter immediately onto slides for 10 minutes. - Shake occasionally.
7. Wash in running water to remove precipitate.
8. Counterstain with 1% Methyl Green for 2 minutes.
9. Wash, air dry slides and mount.

Results:

Chloroacetate esterase activity - blue granules in cytoplasm of granulocytes. Non-specific esterase - dark red granules in monocytes.

Reference: Yam, L.T.; Li, C.Y.; and Crosby, W.H.; American Journal of Clinical Pathology 55; 283-290, 1971. (Slightly modified at NIH by B.J. Coolidge and H.M. Shade)

*Sigma Chemical Company, St. Louis, Missouri 63118

**Cyclo Chemical Corporation, Los Angeles, California 90001

CHROMAFFIN REACTION

Fixation:

Any well fixed adrenal. Zenker's, Helly's or Orth's fixatives preferred.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

3% Potassium Dichromate

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Treat sections in 3% Potassium Dichromate for 3 hours.*
3. Wash in water.
4. Dehydrate in alcohols, clear in xylene and mount.

Results:

Chromaffin substances - brown

Reference: Cowdry, *Laboratory Technique in Biology and Medicine,* pg. 92, 1964.

*Unnecessary if fixed in Zenker's, Helly's or Orth's fixative. For contrast the tissue may be stained with Hematoxylin and Eosin.

COLLOIDAL IRON - Hale - Modification

Fixation:

10% Formalin is preferred (avoid Dichromate fixatives)

Technique:

Paraffin sections cut at 6 microns. Run a control slide.

Prepare:

2% Hydrochloric Acid
2% Potassium Ferrocyanide
Working Colloidal Iron Solution

Acetic acid	5 ml.
Distilled water	15 ml.
COLLOIDAL IRON MÜLLER'S (s)	20 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Rinse briefly (30 seconds) in 12% ACETIC ACID (s).
3. Place in working Colloidal Iron Solution for 1 hour. The control slide is not exposed to Colloidal Iron.
4. Rinse slides in 4 changes of 12% ACETIC ACID (s) for 3 minutes each.
5. Treat for 20 minutes at room temperature in a freshly prepared mixture of equal parts of 2% HCL and 2% Potassium Ferrocyanide.
6. Wash gently in tap water for 5 minutes.
7. Counterstain as desired. We use a regular PAS stain.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:

Mucopolysaccharides - bright blue
The luminal border of the distal tubule system in the kidney and especially the macula densa show a striking concentration of acid material, presumably carbohydrate.

Reference: McManus and Mowry: *Staining Methods - Histologic and Histochemical;* pg. 135-136, 1960.

CONNECTIVE TISSUE - Hematoxylin-Phloxine-Saffron - Masson (Modified)

Fixation:
Zenker-Formol or post-mordant Formalin fixed slides in Bouin's fixative.

Technique:
Cut paraffin sections at 5 microns.

Prepare:
1% PHLOXINE B SOLUTION (s)
Add 1-2 drops of acetic acid to each 100 ml. of 1% Phloxine B Solution.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) for 1 1/2 minutes.
3. Wash well and blue in tepid water.
4. Rinse in distilled water.
5. Stain in 1% Phloxine B Solution for 10 minutes.
6. Wash in running water for 5 minutes to remove excess Phloxine.
7. Wash well in 3 changes of fresh absolute alcohol.
8. Stain in 3.5% SAFRAN DU GATINAIS SOLUTION (s) for 5 minutes.
9. Wash well in three changes of fresh absolute alcohol.
10. Clear in xylene and mount.

Results:
Nuclei - blue
Muscle - red
Collagen - yellow
Bone - orange
Cartilage - yellow to orange
RBC - bright pink
Fibrin - bright red
Mast cell granules - bright pink
Elastic fibers - pale pink

Reference: Masson, Pierre, Diagnostice de Laboratoire, Paris: A Maloine et Fils, 1923. The modification given here was made by Tija Krumins HT (ASCP) of the Lenox Hill Hospital, Laboratory of Pathology in a personal communication.

CONNECTIVE TISSUE - Mallory Heidenhain, Aniline Blue

Fixation:

Any well-fixed tissue. If fixed in Formalin, mordant slides in Bouin's or Zenker's for 1 hour at 60°C.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Working Aniline Blue-Orange G Solution

ANILINE BLUE-ORANGE G SOLUTION (s) ..... 1 part
Distilled water ..... 2 parts

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in AZOCARMINE G SOLUTION (s) at 56°C. for 30 minutes.
3. Rinse in distilled water.
4. Differentiate in ANILINE-ALCOHOL SOLUTION (s) until cytoplasm and connective tissue are pale pink and nuclei stand out sharply.
5. Control differentiation by rinsing in 1% ACETIC ALCOHOL (s).
6. Mordant in 5% PHOSPHOTUNGSTIC ACID SOLUTION (s) for 10-15 minutes.
7. Rinse in distilled water.
8. Counterstain with Working Aniline Blue-Orange G Solution until the finest connective tissue fibers are sharply stained - 15 minutes.
9. Rinse in distilled water.
10. Dehydrate quickly through alcohols.
11. Clear in xylene and mount.

Results:

Chromatin, osteocytes and neuroglia - red
Cytoplasm - pink to blue
Collagen and reticulum - blue
Muscle -red to yellow

Reference: Mallory, F.B., *Pathological Technique,* pg. 154, 1942.

CONNECTIVE TISSUE - Mallory Heidenhain - Knoeff modification

Fixation:

ZF preferred. Mordant other fixatives overnight in 3% potassium dichromate.

Technique:

Cut paraffin sections at 5 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in ANILINE ALCOHOL SOLUTION (s) for 45 minutes.
3. Treat in 1% ACETIC-ALCOHOL SOLUTION (s) for 1-2 minutes.
4. Stain in AZOCARMINE G SOLUTION (s) in 56° oven for 2 hours. (If temperature is too high, Azocarmine differentiates poorly).
5. Rinse slides in distilled water.
6. Differentiate in ANILINE ALCOHOL SOLUTION (s) until cytoplasm and connective tissue are pale pink and nuclei stand out red. Control differentiation by rinsing in 1% ACETIC ALCOHOL (s) and check with microscope. If section is too red, return to Aniline Alcohol until cells are sharply differentiated.
7. Place in 1% ACETIC ALCOHOL (s) for 1-2 minutes.
8. Transfer slides to 5% PHOSPHOTUNGSTIC ACID SOLUTION (s) for 4 hours.
9. Rinse in distilled water.
10. Stain in ANILINE BLUE SOLUTION (s) for 4 hours.
11. Rinse in water.
12. Place in 5% PHOSPHOTUNGSTIC ACID SOLUTION (s) for 15 minutes or until connective tissue is completely decolorized.
13. Rinse in water.
14. Rinse in 1% ACETIC ACID (s) 1-2 minutes.
15. Rinse quickly in 70% alcohol.
16. Dehydrate in alcohols, clear in xylene and mount.

Results:

Chromatin and neuroglia - red
Collagen and reticulum - blue
Muscle - red and yellow
Basophil cytoplasm - light blue
Acidophil cytoplasm - orange-red
Chromophobes - colorless or light gray

Reference: Mallory, F.B., *Pathological Technique,* Philadelphia: W.B. Saunders co., 1938, p. 154.

CONNECTIVE TISSUE - Mallory Heidenhain (Rapid)

Fixation:

ZF preferred, others may be used.

Technique:

Cut paraffin sections at 6 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in MALLORY STAINING SOLUTION (s) for 5 minutes.
3. Wash in running water for 3-5 seconds.
4. Dehydrate rapidly through alcohols. Clear in xylene and mount.

Results:

Collagenous fibrils stain intense blue; ground substances of bone and cartilage, mucus, amyloid and certain other hyaline substances stain varying shades of blue; nuclei, fibroglia, myoglia, and neuroglia fibrils, axis cylinders and fibrin stain red; nucleoli, red blood corpuscles and myelin stain yellow; elastic fibrils stain pale pink or yellow.

Reference: Simpson, Stain Technology 25: 225, 1940. Modification of Mallory Aniline Blue Stain.

CONNECTIVE TISSUE - Pentachrome - Movat

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns.

Prepare:

Alkaline Alcohol
- Ammonium Hydroxide ..... 10.0 ml.
- Alcohol, 95% ..... 90.0 ml.

Crocein Scarlet-Acid Fuchsin Working Solution
- CROCEIN SCARLET-ACID FUCHSIN SOLUTION A (s) ..... 8 parts
- CROCEIN SCARLET-ACID FUCHSIN SOLUTION B (s) ..... 2 parts

Hematoxylin Solution
- HEMATOXYLIN, 10% ALCOHOLIC (s) ..... 25.0 ml.
- Absolute alcohol ..... 25.0 ml.
- FERRIC CHLORIDE, 10% (s) ..... 25.0 ml.
- IODINE - VERHOEFF'S (s) ..... 25.0 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in ALCIAN BLUE (s) for 20 minutes.
3. Wash in running tap water for 5 minutes.
4. Place slides in Alkaline Alcohol for 1 hour.
5. Wash in running tap water for 10 minutes. (Extremely important)
6. Rinse in distilled water.
7. Stain in Hematoxylin Solution for 15 minutes.
8. Rinse in several changes of distilled water.
9. Differentiate in 2% FERRIC CHLORIDE (s). Elastic fibers should be black and background relatively clear.
10. Rinse in distilled water.
11. Place slides in 5% SODIUM THIOSULFATE (s) for 1 minute.
12. Wash in running tap water for 5 minutes.
13. Rinse in distilled water.
14. Stain in Crocein Scarlet-Acid Fuchsin Working Solution for 1 1/2 minutes.
15. Rinse in several changes of distilled water.
16. Rinse in 0.5% ACETIC ACID (s).
17. Place slides in 5% PHOSPHOTUNGSTIC ACID (s) - 2 changes, 5 minutes each.
18. Rinse in 0.5% ACETIC ACID (s).
19. Rinse in 3 changes absolute alcohol.
20. Stain in SAFRAN, ALCOHOLIC 6% (s) for 15 minutes.
21. Rinse in 2 changes absolute alcohol, 2 changes xylene and mount.

Results:

Nuclei and elastic fibers - black
Collagen and reticular fibers - yellow
Ground substance, mucin - blue
Fibrinoid, fibrin - intense red
Muscle - red

Reference: Movat, H.Z.: Arch Path., 60: 289-295, 1955. (Modified by Russell, H.K., 1972)

CONNECTIVE TISSUE - Phosphotungstic Acid-Hematoxylin - One Hour Modification

Fixation

ZF preferred

Technique:

Cut paraffin sections at 4-5 microns.

Staining Procedure:

1. Deparaffinize slides and take through alcohols. Place in IODINE SOLUTION, 0.5% (s) for 10 minutes (regardless of fixative). Proceed through Hypo and into water as usual.
2. Place slides in PTAH SOLUTION (s) at room temperature and transfer to 60°C. for 1 hour. DO NOT PREHEAT.
3. Blot excess stain from slide with bibulous paper and dehydrate quickly in alcohols, clear in xylene and mount.

Results:

Fibrin, nuclei, muscle fibers, keratin and terminal bars - blue.
Collagen, reticular fibers, basement membranes, amyloid, cartilage and thrombocytes - various shades of red.

Reference: Puchtler, Sweat and Doss, Tech. Bull., Vol. 33, 144, 1963.

CONNECTIVE TISSUE - Phosphotungstic Acid Hematoxylin - Mallory - Modified

Fixation:
ZF preferred

Technique:
Cut paraffin sections at 5 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. For other fixatives, mordant sections in a saturated solution of mercuric chloride for 2 hours at 37°C. Remove mercuric chloride crystals as usual.
3. Place in 1% PERIODIC ACID (s) for 2 hours.
4. Rinse in distilled water - 3 changes.
5. Stain in PTAH SOLUTION (s) overnight.
6. Blot slides with bibulous paper, dip quickly in 95% alcohol, dehydrate in absolute alcohols, clear in xylene and mount. Note: Slides should be carried through Step 6 one at a time.

Results:
Fibrin, nuclei, muscle fibers, keratin, and terminal bars - blue
Collagen, reticular fibers, basement membranes, amyloid, cartilage and thrombocytes - various shades of red.

Reference: Mallory, *Pathological Technique,* pg. 76, 1968.

CONNECTIVE TISSUE - Trichrome - Gomori

Fixation:
Any well fixed tissue

Technique:
Paraffin sections cut at 6 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in BOUIN'S SOLUTION (s) at 56°C. for one hour. (omit if Bouin's fixed.)
3. Wash in running water.
4. Stain in MAYER'S HEMATOXYLIN (s) for 1 minute.
5. Wash well and blue in tepid water.
6. Rinse in distilled water.
7. TRICHROME STAIN - GOMORI (s) for 3 minutes.
8. Rinse in distilled water.
9. Differentiate in 0.5% ACETIC WATER (s).
10. Rinse in distilled water.
11. Dehydrate rapidly in alcohols, clear in xylene and mount.

Results:
Collagen - green
Nuclei - blue to black
Striations of muscle are easily demonstrable.

Reference: Am. J. Clin. Path., 20: 661, 1950.

CONNECTIVE TISSUE - Trichrome - Masson - Modified

Fixation:
Any well-fixed tissue

Technique:
Cut paraffin sections at 6 microns

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in 58°C. pre-heated HARRIS HEMATOXYLIN (s) for 5 minutes.
3. Rinse in 95% alcohol.
4. Differentiate in PICRIC ALCOHOL (s) for 10 minutes.
5. Wash in running water for 10 minutes.
6. Stain in PONCEAU RED (s) for 5 minutes.
7. Rinse in distilled water.
8. Differentiate in 1% PHOSPHOMOLYBDIC ACID (s) for 5 minutes.
9. Without rinsing, place in ANILINE BLUE-MASSON (s) for 2 minutes.
10. Rinse in tap water.
11. Place in 1% PHOSPHOMOLYBDIC ACID (s) for 5 minutes.
12. Place in 1% ACETIC ACID (s) for 5 minutes.
13. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nuclei - violet
Collagen - intense blue
Cytoplasm and neuroglia fibrils - vermillion red

Reference: Mallory, *Pathological Technique*, pg. 156, 1968

CONNECTIVE TISSUE - Van Gieson with Celestine Blue

Fixation:
Any

Technique:
Cut paraffin sections at 6 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain sections for 1-5 minutes in CELESTINE BLUE, LENDRUM (s) (examine under microscope at 1 minute intervals.
3. Wash in distilled water.
4. Stain sections for 1/2 to 1 minute in VAN GIESON (s).
5. Pass through 2 changes of absolute alcohol, fairly rapidly, into xylenes and mount.

Results:
Connective tissue and bone - red
Nuclei - black
Cytoplasm - yellow to yellowish green

Reference: Weil, A.: *Textbook of Neuropathology,* 2nd Ed., New York, Grune and Stratton, Inc., p. 330, 1945.

COPPER - Lindquist

Fixation:

10% Neutral Buffered Formalin. Avoid chromates, acid, and unbuffered formalin.

Technique:

Cut paraffin sections at 10 microns. Use distilled water if possible. Do not use pigment removal methods. Use control slide.

Prepare:

Stock Rhodanine Solution (Saturated)

5 p-dimethylaminobenzylidene Rhodanine	0.2 gm.
Absolute alcohol	100 ml.

Working Rhodanine Solution

Stock Rhodanine Solution	6 ml.
Distilled water	94 ml.

(Shake Stock before measuring and mixing)

Diluted Mayer's Hematoxylin

MAYER'S HEMATOXYLIN (s)	50 ml.
Distilled water	50 ml.

0.5% Sodium Borate Solution

Sodium Borate	0.5 gm.
Distilled water	100 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Incubate in Rhodanine Working Solution at 37° for 18 hours.
3. Wash well in several changes of distilled water.
4. Stain in Diluted Mayer's Hematoxylin for 10 minutes.
5. Rinse in distilled water.
6. Rinse quickly in 0.5% Sodium Borate Solution.
7. Rinse thoroughly in distilled water.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:

Copper - bright red
Nuclei - light blue

Reference: Lindquist, R.R., Arch. of Path. 87: No. 4, pp. 370-379, 1969.

DIGESTION BY ENZYMES - Deoxyribonuclease Method

Fixation:
10% Formalin, Bouin's or Carnoy's

Technique:
Cut paraffin sections at 6 microns. Use two slides

Prepare:
Deoxyribonuclease Solution

Deoxyribonuclease, crystalline	0.001 gm.
0.2 M Magnesium Chloride (4.066% $MgC1_26H_20$)	22.5 ml.
0.2 M Calcium Chloride (2.22% $CaC1_2$)	2.5 ml.
0.1 M TRIS BUFFER (s) pH 7.3	20 ml.
Distilled water	55 ml.

Procedure:
1. Deparaffinize slides and hydrate to water.
2. a) Treat one slide in Deoxyribonuclease Solution at 37°C. for 2 hours.
   b) Treat one slide in TRIS BUFFER (s) pH 7.3 at 37°C. for 2 hours.
3. Wash both slides in running water for 10 minutes.
4. Stain as desired. Feulgen Reaction is suggested.

Results:
The tissue elements stained which have been incubated in the Tris buffer and not present in those incubated in the digestion solution are presumably deoxyribonucleic acid.

Reference: Lillie, R.D., *Histopathologic Technic and Practical Histochemistry,* 3rd. Ed., p. 147, 1965.

DIGESTION BY ENZYMES - Diastase Method

see PAS - McManus

DIGESTION BY ENZYMES - Hyaluronidase Method

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns. Use two slides.

Prepare:
Buffer Solution
0.1 M POTASSIUM PHOSPHATE, MONOBASIC SOLUTION (s) ..... 94 ml.
0.1 M SODIUM PHOSPHATE, DIBASIC (s) ..... 6 ml.
Hyaluronidase Digestion Solution
Testicular Hyaluronidase ..... 0.05 gm.
Buffer Solution ..... 100 ml.
Mix just before use.

Procedure:
1. Deparaffinize slides and hydrate to water.
2. a) Treat one slide in Hyaluronidase Solution at 37°C. for one hour.
   b) Treat duplicate slide in Buffer Solution at 37°C. for one hour.
3. Wash both slides in running water for 5 minutes.
4. Stain as desired. Metachromatic stains such as Toluidine Blue between pH 5 and 7 or Colloidal Iron are suggested.

Results:
Hyaluronidase hydrolyzes certain acid mucopolysaccharides such as hyaluronic acid, chondroitin sulfates A and C and sulfuric acid containing polysaccharide of the cornea.
Bacterial hyaluronidase (streptococcal origin) does not hydrolyze the chondroitin sulfates.

Reference: *Manual of Histologic Staining Methods of AFIP*, 3rd. Ed., 1968

DIGESTION BY ENZYMES - Pectinase Digestion Method

(for removing PAS-positive non-glycogen materials from tissues)

Fixation:

10% Formalin

Technique:

Cut paraffin sections at 6 microns. Use two slides.

Prepare:

Pectinase Solution

Pectinase or Pectionol 0	0.4 gm.
Acetate Buffer pH 4.0 - 4.2	100 ml.

Procedure:

1. Deparaffinize duplicate slides and hydrate to water.
2. a) Place test slide in Pectinase Solution for 24 to 48 hours.
   b) Place control slide in acetate buffer pH 4.0 - 4.2 for 24-48 hours.
3. Wash in running water for 10 minutes.

Results:

Pectinase digestion removes mucin, basement membrane, reticulin and many hyaline substances. Collagen does not appear disturbed. Elastica also not disturbed.

Reference: McManus & Mowry, *Staining Methods Histologic and Histochemical*, p. 148, 1960.

DIGESTION BY ENZYMES - Ribonuclease Method

Fixation:

Carnoy's, Telly's or 10% Formalin (Do not use Bouin's)

Technique:

Cut paraffin sections at 6 microns. Use two slides.

Prepare:

Ribonuclease Solution

Ribonuclease, crystalline ..... 0.5 gm.
Distilled water pH 6.8 ..... 100 ml.
Adjust pH with 0.1 N Sodium Hydroxide or Hydrochloric acid.

Procedure:

1. Deparaffinize slides and hydrate to water.
2. a) Treat one slide in Ribonuclease Solution at 37°C. for 3 hours.
   b) Treat one slide in distilled water at 37°C. for 3 hours.
3. Wash in running water for 10 minutes.
4. Stain as desired. Methyl-Green Pyronin is suggested.

Results:

The tissue elements stained which have been incubated in the distilled water and not present in those incubated in the digestion solution are presumably ribonucleic acid.

Reference: McManus and Mowry; *Staining Methods Histologic and Histochemical,* p. 80, 1963.

DIGESTION BY ENZYMES - Sialidase (Neuraminidase) Method

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns. Use two slides.

Prepare:

Buffer Solution #1

1 M Sodium Acetate - 0.4 M Calcium Chloride

Sodium Acetate	27.2 gm.
Calcium Chloride	11.76 gm.
Distilled water	200 ml.

Buffer Solution #2

0.1 M Sodium Acetate - 0.04 M Calcium Chloride

Sodium Acetate	2.7 gm.
Calcium Chloride	1.17 gm.
Distilled water	200 ml.

Sialidase Digestion Solution

Sialidase (neuraminidase)	1 ml.
Buffer Solution #1	0.1 ml.

Procedure:

1. Deparaffinize slides and hydrate to water.
2. Air dry slides for 2 hours. Etch around section to be stained with a diamond pencil.
3. Place slides on glass rods close above layer of water in a petri dish. Place Sialidase Digestion Solution on one slide in sufficient quantity to completely cover section. Cover the control slide with Buffer Solution #2. The petri dishes are then incubated at 40°C. for 3 hours.
4. Remove from incubator and rinse in distilled water.
5. Stain with desired stain. Alcian Blue or Alcian Blue-PAS are suggested.

Results:
Staining attributable to the sialomucins will be selectively eliminated by the sialidase.

Reference: *Manual of Histologic Staining Methods of AFIP,* 3rd. Ed., 1968

DNA - Feulgen - Lillie Modification

Fixation:
Any except Bouin's

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Fast Green Solution
Fast Green ..... 1.01 gm.
95% Alcohol ..... 100 ml.
Sodium Bisulfite Working Solution
1 M SODIUM BISULFITE SOLUTION (s) ..... 10 ml.
Tap water ..... 200 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place in N HYDROCHLORIC ACID (s) at room temperature for 1 minute.
3. Place in pre-heated (60°C.) N HYDROCHLORIC ACID (s) for optimum time of hydrolysis for fixative used.
   Carnoy 3:1 ..... 6 minutes
   Carnoy 6:3:1 ..... 8 minutes
   Champy ..... 25 minutes
   Chrome-acetic ..... 14 mintues
   Flemming ..... 16 minutes
   Formalin ..... 8 minutes
   Helly ..... 8 minutes
   Regaud ..... 14 minutes
   Zenker ..... 5 minutes
   Zenker-Formol ..... 5 minutes
4. Stain with SCHIFF'S REAGENT (s) which has been treated with activated charcoal and filtered, 1/2 - 1 hour.
5. Rinse in 3 changes of Sodium Bisulfite Working Solution for 1-1/2 minutes each. Agitate. Discard after using.
6. Wash in running water for 5 minutes.
7. Counterstain in Fast Green Solution several dips.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nuclear chromatin - deep red-purple
Background - green

Reference: Lillie, R.D., *Histopathologic Technic and Practical Histochemistry,* 3rd. Ed., pp. 148-150, 1965.
Hydrolysis schedule reference - Pearse, A.G.E., *Histochemistry - Theoretical and Applied,* Little, Brown & Co., 1961, p. 823.

DNA - Feulgen - Modification

Fixation:
Any except Bouin's

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Fast Green Solution
Fast Green ..... .01 gm.
95% Alcohol ..... 100.0 ml.

Sodium Bisulfite Working Solution
1 M SODIUM BISULFITE SOLUTION (s) ..... 10.0 ml.
Tap water ..... 200.0 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place in 5N HYDROCHLORIC ACID (s) at room temperature for 1 hour.
3. Stain with SCHIFF'S REAGENT (s) for 45 minutes.
4. Rinse in 3 changes of Sodium Bisulfite Working Solution for 1 1/2 minutes each. Agitate. Discard after using.
5. Wash in running water for 5 minutes.
6. If desired, counterstain in Fast Green Solution several dips.
7. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nuclear chromatin - deep red-purple
Background - green

Reference: Deitch, Wagner and Richart, J. of *Histochemistry and Cytochemistry,* Vol. 16, No. 5, 1968.

ELASTIC FIBERS - Orcein - Taenzer-Unna

Fixation:
Any

Technique:
Cut paraffin sections at 6 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Warm ORCEIN STAINING SOLUTION (s) 10-15 minutes. Place slides in solution at room temperature for 1 hour.
3. Wash thoroughly in 70% alcohol.
4. Wash in water to fix color.
5. May be counterstained with MAYER'S HEMATOXYLIN (s) for 30 seconds. Blue in tap water.
6. Dehydrate in 95% alcohol and absolute alcohols.
7. Clear in xylene and mount.

Results:
Elastic fibers - deep rich brown
Connective tissue - pale brown
Nuclei - blue to black

Reference: Mallory, *Pathological Technique,* pg. 171, 1968.

ELASTIC FIBERS - Verhoeff's Van Gieson

Fixation:
10% Formalin or Zenker's

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Verhoeff Hematoxylin

5% ALCOHOLIC HEMATOXYLIN (s)	20 ml.
10% FERRIC CHLORIDE (s)	8 ml.
VERHOEFF IODINE (s)	8 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in Verhoeff Hematoxylin Solution for 15 minutes - 1 hour.
3. Wash in distilled water.
4. Differentiate in 2% FERRIC CHLORIDE (s). This requires only a few minutes. Examine sections in water under low magnification.
5. Wash in water.
6. Place in 95% alcohol for 5 minutes.
7. Wash in distilled water for 5 minutes.
8. Counterstain in Van GIESON (s) for 30 seconds to 1 minute.
9. Dehydrate rapidly through 2 absolute alcohols, clear in xylene and mount.

Results:
Elastic fibers - blue-black to black
Nuclei - blue to black
Collagen - red
Other tissue elements - yellow
Degenerated elastic tissue (elastin) is also stained by this method.

Reference: Verhoeff, F.H., J.A.M.A., 50:876, 1908.

EOSINOPHILS - Modified Dominici Stain

Fixation:
Bouin's

Technique:
Cut paraffin sections at 4 microns.

Prepare:

Sorensen's Buffer pH 6.2
- M/15 POTASSIUM PHOSPHATE BUFFER (s) ..... 252 ml.
- M/15 SODIUM PHOSPHATE (dibasic) BUFFER (s) .... 48 ml.

Orange G-Eosin Y Solution
- Orange G ..... 0.5 gm.
- Eosin Y ..... 0.5 gm.
- Sorenson's Buffer pH 6.2 ..... 100 ml.

Toluidine Blue Solution
- Toluidine Blue O ..... 0.3 gm.
- Sorenson's Buffer pH 6.2 ..... 100 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain slides in Orange G-Eosin Y Solution for 5 minutes.
3. Rinse quickly in Sorenson's Buffer pH 6.2.
4. Counterstain with Toluidine Blue Solution for 5-30 seconds.
5. Rinse quickly in Sorenson's Buffer pH 6.2
6. Differentiate in 95% alcohol.
7. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Eosinophils - bright orange granules
Neutrophils - lilac granules
RBC - pink
Nuclei - various shades of blue and violet
Cytoplasm of plasma cells - intensely blue

Reference: Dominici, M.: C. rend. Soc. biol., 54: 221, 1902.

ESTERASE

Fixation:

Cold 10% Formalin. For smears fix for 2 minutes in cold 10% Formalin made in methanol. Enxymes are heat labile, pH dependent and diffuse in water. For bone specimens Versenate (EDTA) decalcification should be used to preserve enzyme activity. Esterase may be demonstrated with mercuric chloride fixative such as B-5 but sections must not be treated with iodine and hypo to remove mercuric chloride crystals. Iodine-hypo inhibits staining.

Technique:

Cut paraffin sections at 5 microns. Tissue must be infiltrated with paraffin as quickly as possible and not subject to temperature above 60°C. Run positive control slide. Source of control - liver and kidney.

Prepare:

Solution A

Concentrated Hydrochloric acid	1.6 ml.
Distilled water	8.4 ml.
p-Rosanilin, acridin-free	0.4 gm.

Solution B

Sodium Nitrite	0.4 gm.
Distilled water	10 ml.

Solution C

0.1N HYDROCHLORIC ACID (s)	14.6 ml.
0.1M SODIUM BARBITAL (s)	15.4 ml.

Solution D

Naphthol AS-D Chloroacetate	0.01 gm.
N, N-Dimethylformamide	1.0 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Mix one drop of Solution A and one drop of Solution B in a 50 ml. beaker and let stand for 1 minute.
3. Add Solution C and mix well. **°Adjust pH to 6.3.**
4. To Solution D, add the solution from Step 3, mix well and filter. Immediately place slides in this solution for 30 minutes. If solution turns pink upon mixing - discard and repeat.
5. If, after 30 minutes, the reaction is not complete, re-filter the solution and replace slides for another 30 minutes.
6. Wash slides in running water for 5 minutes.
7. Counterstain in MAYER'S HEMATOXYLIN (s) for 5 minutes.
8. Air dry.
8. Clear in xylene and mount.

Results:

Esterase - red
Nuclei - blue

Reference: Leder, L.D., Selective Enzymocytochemical Demonstration of Neutrophil Myeloid Cells and Tissue Mast Cells in Paraffin Sections. Klinische Wochenschrift, V. 42, No. 11, 553, 1964.

*If pH is above 6.3 adjust with 0.1N HC1 (s).
If pH is below 6.3 adjust with 0.1M SODIUM BARBITAL (s).

FAT AND FATTY ACIDS - Nile Blue Sulfate

Fixation:

10% Formalin. Do not transfer to alcohol.

Technique:

Cut frozen sections at 10 microns.
Fat is removed by alcohol and heat.

Staining Procedure:

1. Place sections in distilled water.
2. Stain in NILE BLUE SULFATE SOLUTION (s) for 20 minutes.
3. Rinse in tap water.
4. Differentiate in 1% ACETIC ACID (s) until the pink color is clear. Check under the microscope.
5. Wash thoroughly in distilled water.
6. Mount in glycerin jelly.

Results:

Neutral fats - pink
Fatty acids - blue to violet
Nuclei and elastic tissue - dark blue
Note: There is a question as to whether neutral fats and fatty acids are differentiated by this method, but it is included because it may be of value in some instances.

Reference: M.F. Gridley's Modification of Smith's fat stain used at Armed Forces Institute of Pathology (Smith L., J. Path. and Bact., 12: 1, 1907).

FAT - Oil Red O

Fixation:

10% Formalin - Do not transfer to alcohol.

Technique:

Cut frozen sections at 10-12 microns. Fat is removed by alcohol and heat.

Prepare:

Oil Red O Working Solution

OIL RED O SOLUTION (s) ..... 60 ml.
Distilled water..... 40 ml.
Shake vigorously and let stand for 10 minutes. Filter and use immediately.
Note: Warm GLYCERIN GELATIN MOUNTING MEDIA (s) in a water bath until melted.

Staining Procedure:

1. Rinse free-floating sections in 3 changes of distilled water.
2. Transfer sections to a small dish containing COOLIDGE FROZEN SECTION CLARIFIER* (s) for 5 seconds.
3. Rinse sections in distilled water and mount on albuminized slides.
4. Dry in hot air oven at 60°C. for 10 minutes.
5. Pour freshly filtered Oil Red O Working Solution into coplin jar, place slides in jar and cover. Stain for 8 minutes.
6. Rinse slides in 3 changes of distilled water.
7. Counterstain with MAYER's HEMATOXYLIN (s) for 2 minutes.
8. Place gently in warm water until nuclei are blue.
9. Rinse in distilled water.
10. Mount in warm GLYCERIN GELATIN MOUNTING MEDIA (s).

Results:

Fat - orange to deep red
Nuclei - blue

Reference: Modification of Lillie and Ashburn, Arch. Path., 36: 432-436, 1943.
*This solution clears the tissue and improves the flatness and adherence of sections to slides.
Stain Tech., May 1972.

FIBRIN - Lendrum Acid Picro - Mallory

Fixation:
Any

Technique:
Cut paraffin sections at 6 microns

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in CELESTINE BLUE-LENDRUM (s) 3-5 minutes.
3. Rinse in tap water.
4. Stain in MAYER'S HEMATOXYLIN (s) 5 minutes.
5. Wash in running water 5 minutes.
6. Rinse in 95% alcohol.
7. Stain in PICRO-ORANGE (s) 2 mintues.
8. Stain in ACID FUCHSIN (s) 5 minutes.
9. Rinse in distilled water.
10. Rinse briefly in equal parts PICRO-ORANGE (s) and 80% alcohol.
11. Differentiate in 1% PHOSPHOTUNGSTIC ACID(s) for 5-10 minutes until colors are clear.
12. Rinse in distilled water.
13. Stain in ANILINE BLUE-MASSON (s) 2-10 mintues.
14. Rinse in distilled water 2 minutes.
15. Dehydrate in alcohols, clear in xylene and mount.

Results:
Fibrin - red
Erythrocytes - orange
Collagen - blue
Nuclei - blue-black

Reference: Humason, G.L., *Animal Tissue Techniques,* 3rd. Ed., p. 266, 1972, Freeman and Co.

FUNGI - FLUORESCENT - Pickett

Fixation:

10% Formalin, Zenker's, Alcohol and other general fixatives.

Technique:

Cut paraffin sections at 5-6 microns. Use gelatinized slides and control slide.

Prepare:

Weigert's Working Solution

Equal parts of WEIGERTS IRON HEMATOXYLIN SOLUTION A (s) AND SOLUTION B (s).

Acridine Orange Solution

Acridine Orange ..... 1 gm.
Distilled water ..... 100 ml.

Acridine Orange Solution may be stored in a brown bottle in the refrigerator. Do not expose to fluorescent lights.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in Weigert's Working Solution for 5 minutes.
3. Wash in running water for 5 minutes.
4. Stain in Acridine Orange Solution for 2 minutes.
5. Rinse in water for 30 seconds.
6. Dehydrate in alcohols, clear in xylene and mount in a nonfluorescing medium.

Results:

All fungi fluoresce except Nocardia and Rhizopus colors of the fluorescing genera is as follows:

Coccidioides, Rhinosporidium - red
Aspergillus - green
Actinomyces, Histoplasma - red to yellow
Candida, Blastomyces dermatitids, Monosporium - yellow-green
Blastomyces brasiliensis - yellow

Old hematoxylin and eosin slides may be decolorized and re-stained as above. Weigert hematoxylin staining acts as a quenching agent for the background.

Reference: Pickett, J.P.; Bishop, C.M.; Chick, W.E.; and Baker, R.D., Am. J. Clin. Path., 34: 197-202, 1960.

FUNGI - Gridley - Lillie Modification

Fixation:

10% Formalin

Technique:

Cut paraffin sections at 6 microns. Use positive control slide.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Oxidize in 4% CHROMIC ACID SOLUTION (s) for 1 hour.
3. Wash in running water for 5 minutes.
4. Place in SCHIFF'S REAGENT (s) for 15 minutes.
5. Wash in running water for 15 minutes.
6. Rinse in several changes of 70% alcohol.
7. Stain in ALDEHYDE FUCHSIN - GOMORI (s) for 30 minutes.
8. Rinse in 95% alcohol.
9. Rinse in distilled water.
10. Counterstain with METANIL YELLOW SOLUTION (s) for 1 minute.
11. Rinse in distilled water.
12. Dehydrate in alcohols, clear in xylene and mount.

Results:

Mycelia - deep purple
Candida - deep rose to purple
Elastic tissue and mucin - deep purple
Background - yellow
Filaments of Nocardia and Actinomyces are not stained.

Reference: Gridley, M.F.: Amer. J. Clin. Path. 23: 303-307, 1953

FUNGI - Methenamine - Grocott

Fixation:

10% Formalin

Technique:

Cut paraffin sections at 6 microns. Use control slide and chemically clean glassware.

Prepare:

Silver Nitrate Working Solution

METHENAMINE-SILVER SOLUTIN (s)	25 ml.
5% BORAX (SODIUM BORATE) (s)	2 ml.
Distilled water	23 ml.

Light Green Working Solution

0.1% LIGHT GREEN (s)	10 ml.
Distilled water	30 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Oxidize in 5% CHROMIC ACID (s) for 1 hour.
3. Wash in running water for 10 minutes.
4. Treat in 1% SODIUM BISULFITE (s) for 1 minute.
5. Wash in running water for 5 minutes and rinse in 3 changes of distilled water.
6. Place in Silver Nitrate Working Solution at 40-50°C. for 1 hour. Check control at 30 minutes. Section tan, fungi dark brown to black.
7. Rinse in 3 changes of distilled water.
8. Tone in 0.1% GOLD CHLORIDE (s) for 1-2 minutes.
9. Rinse in distilled water.
10. Treat in 2% SODIUM THIOSULFATE (s) for 1-2 minutes.
11. Wash in running water for 5 minutes.
12. Counterstain in Light Green Working Solution.
13. Dehydrate in alcohols, clear in xylene and mount.

Results:

Fungi - sharply delineated in black with inner parts of mycelia and hyphae stained old rose.
Mucin - rose-red
Background - pale green

Reference: Grocott, R.G., Am. J. Clin. Path., 25: 575-579, 1955.

GIEMSA FOR ASCITES AND BLOOD CELL CULTURES

Fixation:

Fix smears in Methyl Alcohol for 5 minutes.

Technique:

Smears of concentrated ascites fluid or blood cells from cultures.

Prepare:

Giemsa Solution

Distilled water	46 ml.
GIEMSA (s)	2 ml.
SODIUM PHOSPHATE M/15 DIBASIC (s)	1 ml.
POTASSIUM PHOSPHATE M/15 (s)	1 ml.

(These are Sorenson's buffers)

Staining Procedure:

1. Stain in Giemsa solution 8 minutes.
2. Wash in distilled water.
3. Air dry, dip in xylene and mount.

Results:

Nuclei - dark blue
Cytoplasm - light blue

Reference: Dr. Louis Epstein's modification of Giemsa Stain (formerly NIH)

GIEMSA FOR BLOOD SMEARS - May-Grunwald

Fixation:
Methyl Alcohol - 10 minutes

Technique:
Blood smears

Prepare:
Giemsa Working Solution
GIEMSA (s) ..... 50 drops
McJUNKIN-HADEN BUFFER (s) ..... 50 ml.

Staining Procedure:
1. Flood slides with MAY-GRUNWALS (s) for 3 minutes.
2. Add an equal volume of McJUNKIN-HADEN BUFFER (s) for 1 minute.
3. Wash with buffer.
4. Stain with Giemsa Working Solution for 10 minutes.
5. Wash in running water and rinse in distilled water.
6. Air dry, dip in xylene and mount.

Results:
Nuclei - blue
Cytoplasm - pink to rose
Bacteria - blue

Reference: A Modification of the Pappenheim Technique. Folia, Haematol., Arch., 22: 15, 1917.

GIEMSA FOR LYMPHOCYTE CULTURES

- Radioautographs

Fixation:
Methyl Alcohol

Technique:
Smears on gelatinized slides. Dip in emulsion and develop.

Prepare:

Working Buffer Solution

0.1 M CITRIC ACID (s) ..... 85 ml.
0.2 M SODIUM PHOSPHATE (DIBASIC) (s) ..... 115 ml.
Make up to 1000 ml. with distilled water and adjust pH to 5.75.

Giemsa Staining Solution

Working Buffer Solution ..... 400 ml.
Methyl alcohol ..... 15 ml.
GIEMSA (s) ..... 25 ml.
Bring volume to 500 ml. with Working Buffer Solution.

Staining Procedure:
Three minutes in each solution.

1. 50% Methyl alcohol -50% Working Buffer Solution.
2. 95% Methyl alcohol - 5% Working Buffer Solution.
3. Working Buffer Solution.

For steps 4, 5, and 6 -repeat steps 1, 2, and 3.

7. Working Buffer Solution - 2 changes.
8. Allow slides to dry.
9. Stain with Giemsa Staining Solution for 8 minutes.
10. Rinse with Working Buffer Solution.
11. Allow slides to dry.
12. Rinse in xylene and mount.

Results:
Lymphocytes -blue
Silver grains - black

Reference: Personal communication, Dr. Louis Epstein - formerly NIH.

GIEMSA FOR PARAFFIN SECTIONS - May-Grunwald

Fixation:
Any

Technique:
Paraffin sections cut at 6 microns or smears fixed in methyl alcohol.

Prepare:
Working Giemsa Solution

GIEMAS SOLUTION (s)	5 ml.
Distilled water	95 ml.

Staining Procedure:

1. Deparaffinize sections in xylene and bring to absolute alcohol.
2. Place sections in absolute methyl alcohol, two changes for 3 minutes each.
3. Place in freshly prepared mixture of equal parts of MAY-GRUNWALD solution (s) and distilled water for 6 minutes.
4. Place directly into Giemsa solution for 45 minutes.
5. Wash sections in distilled water.
6. Differentiate in 95% alcohol to which has been added a few drops of acetic acid until no more color comes out.
7. Wash in fresh 95% alcohol. Dehydrate in absolute alcohols.
8. Clear in xylene and mount.

Results:
Nuclei - blue
Cytoplasm - pink to rose
Bacteria - blue

Reference: Strumia, M.M.: J. Clin. Meth., 21: 930-934, 1935-1936.

GIEMSA FOR DIFFICULT TO STAIN PARAFFIN SECTIONS

Fixation:

10% Formalin or Zenker's

Technique:

Cut paraffin sections at 6 microns. Cut spleen, lymph nodes, thymus, etc. at 4-5 microns.

Prepare:

Giemsa Working Solution for Zenker Fixation

GIEMSA STOCK (s) ..... 6.1 ml.
Make up to 100 ml. with distilled water.
Add M/15 CITRIC ACID-METHYL ALCOHOL BUFFER (s) ..... 4 ml.
M/15 SODIUM PHOSPHATE-METHYL ALCOHOL BUFFER (s) ..... 4 ml.
pH 4.1 (equal parts acid and base buffers)

Giemsa Working Solution for Formalin Fixation

GIEMSA STOCK (s) ..... 6.1 ml.
Make up to 100 ml. with distilled water.
Add M/15 CITRIC ACID-METHYL ALCOHOL BUFFER (s) ..... 5.33 ml.
M/15 SODIUM PHOSPHATE-METHYL ALCOHOL BUFFER (s) ..... 2.67 ml.
pH 3.62 (3:2 mixture of acid and base buffers)
Do not filter working solution.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in appropriate Giemsa Working Solution overnight at room temperature.
3. Differentiate in 95% alcohol, dehydrate in absolute alcohols.
4. Clear in xylene and mount.

Results:

Nuclei - blue
Cytoplasm - pink to rose
Bacteria - blue

GLYCOGEN - PAS - Hotchkiss - Modified for Blood Smears

Fixation:
Methyl Alcohol*

Technique:
Blood smears

Prepare:
Reducing Bath
Distilled water ..... 100 ml.
1 N HYDROCHLORIC ACID (s) ..... 5 ml.
10% SODIUM BISULFITE (s)
See: SULFUROUS ACID RINSE, 10% (s)..... 6 ml.
(The solution should smell strongly of sulfur dioxide)
Celestine Blue Solution
Distilled water ..... 100 ml.
Ferric Ammonium Sulfate ..... 5 gm.
Hydrochloric acid ..... 0.5 ml.
Heat to just under 100°C. and add
Celestine Blue ..... 0.5 gm.
Filter before use. The solution is good for 2-3 months.

Staining Procedure:
1. Place slides in 0.5% PERIODIC ACID SOLUTION (s) for 7 minutes.
2. Rinse carefully in distilled water.
3. Place slides in reducing bath for 1 minute.
4. Rinse in distilled water.
5. Place in SCHIFF'S REAGENT (s) for 30 minutes.
6. Wash in running water for 5-10 minutes.
7. Rinse in distilled water.
8. Stain in filtered Celestine Blue Solution for 10-15 minutes.
9. Rinse in distilled water and air dry.
10. Dip in xylene and mount.

Results:
Glycogen - rose to purplish red
Background - blue

Reference: Hotchkiss, R.D., Arch. Biochemistry 16, 131-142, 1948.
*Glycogen is water soluble.

GLYCOGEN - PAS - McManus

Fixation:

Any well-fixed tissue. For glycogen, alcoholic fixatives are preferred.* If fixed in gluteraldehyde, tissue must be treated in: Acetic Acid - 40 ml. Aniline oil - 4 ml. for 1-2 hours. Wash in water and continue with Step 2.

Technique:

Cut paraffin sections at 6 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in 0.5% PERIODIC ACID (s) for 10 minutes.
3. Rinse in distilled water.
4. Place in SCHIFF'S REAGENT (s) for 15 minutes. (Bring solution to room temperature before use.)
5. Place in 2 changes of SULFUROUS ACID RINSE (s) Working Solution for 3 minutes each.
6. Wash in running water for 5 minutes and rinse in distilled water.
7. Stain lightly with MAYER'S HEMATOXYLIN (s) for 30-45 seconds.
8. Place in warm water until nuclei are blue and rinse in distilled water.
9. Dehydrate in alcohols, clear in xylene and mount.

Results:

Reitculum - purple-red
Glycogen - deep purple-red
Mucin - red to purple
Yeast and mold chitin - red
Fibrin and collagen - pink

Reference: McManus, J.F., Stain Technology, 23:99-108, 1948.

If the presence of glycogen is to be proven, a diastase digestion can be done in conjunction with the PAS.
Note: Extra slides should be used because sections tend to fall off the slides. Celloidin cannot be used.

Prepare:

Diastase Solution

DIASTASE SOLVENT (s) ..... 100 ml.
Diastase of malt ..... 0.1 gm.
Diastase of malt should be gently sprinkled on surface of solvent.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in Diastase Solution for 1 hour at 37°C. or 2 hours at room temperature.
3. Wash in running water, rinse in distilled water.
4. Proceed with PAS from Step 2.

Results:

Diastase removes glycogen.

Reference: Lillie, R.D., Stain Technology, Vol. 22, No. 2, April, 1947.

*Glycogen is water soluble. Fixation of tissue must take place within 10 minutes after removal from animal because glycogen is extremely labile to post mortem autolytic changes.

GLYCOGEN - Best's Carmine - Lillie modification

Fixation:
Absolute Alcohol preferred.

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Working Carmine Solution
BEST'S CARMINE SOLUTION (s) freshly filtered ..... 20 ml.
Ammonium Hydroxide (28%)..... 30 ml.
Then add drop by drop, with constant agitation
Methyl alcohol ..... 30 ml.
Differentiation Solution
Ethyl alcohol..... 80 ml.
Methyl alcohol ..... 40 ml.
Distilled water ..... 100 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain deeply with MAYER'S HEMATOXYLIN (s) 3 minutes.
3. Wash in water.
4. Stain in Working Carmine Solution for 20 minutes (agitate frequently).
5. Transfer directly into differentiating solution, two changes - 1 dip each.
6. Rinse quickly in 80% alcohol.
7. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Glycogen - red
Nuclei - blue

Reference: Mallory, F.B., *Pathological Technique,* pp. 126-129, 1961.

GOLGI - DaFano

Fixation:

Cobalt Nitrate. Fix 3 specimens, not exceeding 1 mm. in thickness

Technique:

Cut paraffin sections at 2-4 microns. Use chemically clean glassware. Avoid metal. Tissues are impregnated with silver before they are embedded.

Prepare:

Fixative Solution

Cobalt Nitrate	1 gm.
Distilled water	100 ml.
Formaldehyde 37-40%	15 ml.

1.5% Silver Nitrate Solution

Silver Nitrate	4.5 gm.
Distilled water	300 ml.

Cajal's Reducing Solution

Hydroquinone	1 gm.
NEUTRAL FORMALIN (s)	7.5 ml.
Distilled water	50 ml.
Sodium Sulfite	0.25 gm.

This solution must be freshly made.

Staining Procedure:

1. Place 3 specimens in Fixative Solution for 2-4 hours.
2. Rinse in distilled water.
3. Place specimens in a large volume (300 ml.) of Silver Nitrate Solution in the dark for 16-24 hours.
4. Rinse in several changes of distilled water.
5. Place the 3 specimens in the Cajal's Reducing Solution. Remove the first after 8 hours, the second after 16 hours and the third after 24 hours.
6. Dehydrate, clear and infiltrate tissues in the usual manner on the tissue processor and embed in paraffin.
7. Cut sections at 2-4 microns.
8. Deparaffinize in several changes of xylene and mount.

Results:

Golgi elements - black
Background - yellow to golden brown

Reference: DaFano, C.: J. Micr. Soc., 157-161, 1920.

HEMATOXYLIN AND EOSIN

1. Xylene - 2 changes ..... 2-3 minutes each
2. Absolute alcohol - 2 changes ..... 2-3 minutes each
3. 95% alcohol - 1 change ..... 2-3 minutes
4. IODINE SOLUTION 0.5% (s) ..... 3 minutes
5. 70% alcohol ..... several dips
6. HYPO (s) ..... 3 minutes
   (steps 4, 5, and 6 are necessary only when tissues are fixed in a mercuric chloride solution).
7. Wash in running tap water.
8. Rinse well in distilled water.
9. MAYER'S HEMATOXYLIN (s) ..... 30 seconds - 2 minutes
10. Rinse in running tap water to remove excess stain and place in lukewarm water to "blue" sections.
    (If slides stay in water for a long period, we have found that the chlorine will bleach the hematoxylin).
11. Rinse in distilled water.
12. 50% alcohol ..... 1-2 minutes
13. 70% alcohol ..... 1-2 minutes
14. 0.5% ALCOHOLIC EOSIN (s) or EOSIN AND PHLOXINE B* (s) slightly
    acidified with glacial Acetic acid ..... 5 dips - 30 seconds
15. 95% alcohol - 3 changes ..... 30 seconds each
    During these steps the slides are agitated to remove excess eosin. If this is not done the overlay of the eosin gives the sections a muddy appearance. The 95% alcohol is changed frequently.
16. Absolute alcohol - 2 changes ..... 1-2 minutes each
17. Alcohol -xylene ..... agitate - 30 seconds
    If sections look milky, dehydration is not complete. Take back to clean absolute alcohol and return to fresh alcohol-xylene.
18. Xylene - several changes until sections are clear.
19. Mount.

*EOSIN AND PHLOXINE B (s)

1% Eosin Y	100 ml.
1% Phloxine B	10 ml.
95% Alcohol	780 ml.
Acetic Acid	4 ml.

Results:

Nuclei - blue
Cytoplasm - pink

HEMOFUSCIN - Mallory

Fixation:

Zenker's, Absolute Alcohol or 10% Formalin

Technique:

Cut paraffin sections at 6 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) for 2 minutes.
3. Wash well and blue in tepid water.
4. Stain in 0.5% BASIC FUCHSIN ALCOHOLIC SOLUTION (s) for 30 minutes.
5. Wash in running water for 5 minutes.
6. Differentiate in 95% alcohol until red hemofucsin granules stand out sharply.
7. Dehydrate in alcohols, clear in xylene and mount.

Results:

Hemofuscin granules - bright red
Nuclei -blue

Reference: Mallory, F.B., *Pathological Technique*, pg. 136, 1961.

HEMOGLOBIN - Dunn

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns

Prepare:
Working Solution Leuco-Patent Blue
LEUCO-PATENT BLUE SOLUTION (s) ..... 20 ml.
Acetic acid..... 4 ml.
3% Hydrogen Peroxide..... 2 ml.
(30% Hydrogen Peroxide diluted 1-9 with distilled water.)

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place slides in Working Solution Leuco-Patent Blue for 5 minues.
3. Rinse quickly in distilled water.
4. Counterstain with EOSIN (s) for 1 minute.
5. Dehydrate in alcohols, clear in xylene and mount.

Results:
Hemoglobin dark blue or blue green
Leucoyte Granules - dark blue
Background - pink

Reference: Pearse, *Histochemistry, Theoretical and Applied,* 2nd Ed. p. 904, 1961.

HEMOGLOBIN AND HEMOSIDERIN -
Puchtler-Sweat Method

Fixation:
Zenker-Formol (other fixatives mordant slides in ZF overnight)

Technique:
Cut paraffin sections at 6 microns. Avoid use of metal.

Prepare:

2% Potassium Ferrocyanide Solution

2% Hydrochloric Acid Solution

Methanol-Glacial Acetic Acid Solution

Methyl alcohol	90 ml.
Acetic acid	10 ml.

Potassium Ferrocyanide-Hydrochloric Acid Solution

2% Potassium Ferrocyanide Solution	50 ml.
2% Hydrochloric Acid Solution	50 ml.

(Mix just before use)

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Rinse thoroughly in distilled water.
3. Place slides in Potassium Ferrocyanide-Hydrochloric Acid Solution for 30 minutes.
4. Rinse in 3 changes of distilled water.
5. Place in 5% TANNIC ACID SOLUTION (s) for 10 minutes.
6. Rinse in 3 changes of distilled water.
7. Place in 1% PHOSPHOMOLYBDIC ACID SOLUTION (s) for 10 minutes.
8. Rinse in 3 changes of distilled water.
9. Stain in PHLOXINE B SOLUTION (s) pH 3.7 for 5 minutes. Do not filter.
10. Rinse in 2 changes of Methanol-Glacial Acetic Acid Solution.
11. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Hemoglobin - red
Hemosiderin - dark blue to blue-green

Reference: Puchtler, H. and Sweat, F.: Arch. Path., 75:588-590, 1963.

HISTIDINE - Pauly's Reaction

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 8 microns

Prepare:
Pauly's Solution
.05 gm. Sulfanilic acid in 5 ml. of 3N HYDROCHLORIC ACID (s) ..... 2 ml.
5% SODIUM NITRITE (s) ..... 2 ml.
Distilled water ..... 20 ml.
Chill to 15°C. stir for 10 minutes.
Add 20% SODIUM CARBONATE (s) ..... 6 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain with Pauly's Solution for 4 minutes.
3. Rinse in 2 changes of 70% alcohol to which has been added a few drops of 20% SODIUM CARBONATE (s).
4. Dehydrate in alcohols, clear in xylene and mount.

Results:
Histidine positive - orange-red

Reference: Reaven, E.P. and Cox, A.J., Histochemistry and Cytochemistry, Vol. 11, 782, 1963.

INCLUSION BODIES - Lendrum

Fixation:
10% Formalin or Zenker's

Technique:
Cut paraffin sections at 6 microns

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) for 5 minutes.
3. Wash in running water until blue.
4. Stain in PHLOXINE LENDRUM SOLUTION (s) for 30 minutes.
5. Rinse quickly in distilled water and drain.
6. Place slides in TARTARZINE SOLUTION (s). (Shake solution before pouring from stock bottle). This solution decolorizes the phloxine and stains background yellow.
7. Dehydrate in 60%, 95% and absolute alcohols, clear in xylene and mount.

Results:
Inclusion bodies - red
Nuclei - blue
Background - yellow
Paneth cells -pink

Reference: Lendrum, A.C.: J. Path. and Bact., 59: 399-404, 1947.

IRON - Gomori

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns. Use control slide. Avoid metal until after Step 3.

Prepare:

Solution A

Hydrochloric acid	20 ml.
Distilled water	80 ml.

Solution B

Potassium Ferrocyanide	10 gm.
Distilled water	100 ml.

Solution C

BASIC FUCHSIN - SATURATED (s)	10 drops
Distilled water	50 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in a freshly prepared mixture of equal parts of Solution A and Solution B - 20 minutes.
3. Wash thoroughly in distilled water.
4. Counterstain with Solution C - 30 seconds.
5. Differentiate and dehydrate in 80%, 95% and absolute alcohols.
6. Clear in xylene and mount.

Results:
Iron - blue
Other elements - pink to red

Reference: Gomori, G., Amer. J. Path., 12: 655-663, 1936.

JUXTAGLOMERULAR GRANULES - Bowie

Fixation:
Helly's

Technique:
Cut paraffin sections at 4 microns. Excess albumin may interfere with staining.

Prepare:
Stock Bowie Solution
BOWIE STAIN PRECIPITATE (s) ..... 0.2 gm.
95% Ethyl alcohol ..... 20 ml.
Working Bowie Solution
Stock Bowie Solution ..... 10-15 drops
20% Ethyl alcohol ..... 100 ml.
2.5% Potassium Dichromate

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
   (Do not leave in Iodine or Hypo over 3 minutes each).
2. Mordant in 2.5% Potassium Dichromate at 37° overnight.
3. Rinse in water.
4. Place sections in Working Bowie Solution overnight.
5. Blot with bibulous paper and dip quickly in 2 changes of acetone to remove excess stain.
6. Differentiate in equal parts of xylene and clove oil until sections appear magenta or reddish-purple grossly. Differentiations should be checked with the microscope.
7. Clear in 3 changes of xylene and mount.

Results:
Renal parenchyma - red (or magenta)
Elastic tissue of vessels - blue purple
Granulated juxtaglomerular cells - blue-purple
Red blood cells - amber

Reference: Pitcock, J.A. and Hartroft, P.M.; Am. J. Path., 34: 863-864, 1958.

JUXTAGLOMERULAR GRANULES - Smith

Fixation:
Smith's Fixative

Technique:
Cut paraffin sections at 6 microns

Prepare:

Bowie Solution

BOWIE PRECIPITATE (s)	0.2 gm.
Absolute Alcohol	20.0 ml.

Ziehl's Carbol-Fuchsin

Basic Fuchsin	1.0 gm.
Phenol crystals	5.0 gm.
Grind in a mortar; add while grinding - 90% alcohol	10.0 ml.

in 1 ml. portions.
Then: 100 ml. distilled water is added in 10 ml. lots to wash mortar.
Accumulated washings are filtered for use.
Solution may be used for 1-2 weeks.

1% Potassium Permanganate

1% Sulfuric Acid

Potassium Permanganate Solution

1% Potassium Permanganate	50.0 ml.
1% Sulfuric Acid	50.0 ml.

Acid Alcohol Solution

35% Alcohol	100.0 ml.
Hydrochloric Acid	0.5 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Oxidize in Potassium Permanganate Solution for 1 minutes.
3. Rinse briefly in distilled water.
4. Decolorize in 1% SODIUM BISULFITE SOLUTION (s) for 1-3 minutes or until sections are no longer brown.
5. Wash in running water for 2 minutes.
6. Stain in Ziehl's Carbol-Fuchsin for 1 minute.
7. Wash in running water for 1 minute.
8. Decolorize in Acid Alcohol Decolorizing Solution until sections are pink. Retention of red color in some nuclei is more desirable than complete extraction.
9. Wash in running water for 2 minutes.
   Handle each slide individually in subsequent steps.
10. Place slide flat on staining rack without draining or shaking off surface water. Drop on sufficient Bowie Solution to cover sections without flooding the slide. After 5-10 seconds immerse the slide in a coplin jar of water without draining dye from slide. Wait 1-2 minutes before washing in water.
11. Dehydrate rapidly in two changes of acetone and one 1:1 acetone-xylene.
12. Clear in xylene and mount.

Results:

Juxtaglomerular granules - deep purple
Nuclei - majority lavendar, some pink to red
Cytoplasm - shades of blue or purple
Elastic tissue - deep purple
Collagen - pink
Erythrocytes - red
In mouse and rat mast cell granules, beta cell granules in pancreas and pituitary, zymogen granules in gastric mucosa and pancreas, paneth cell granules, and secretion granules in submandibular salivary gland are sharply demonstrated.

Reference: Smith, Carlton L., Stain Technology, Vol. 41, No. 5, 291-294, 1966.

JUXTAGLOMERULAR GRANULES - Thioflavine T

Fixation:
10% Formalin preferred. ZF may be used.

Technique:
Cut paraffin sections at 4-6 microns. Use gelatinized slides.

Prepare:
1% Thioflavine T
Thioflavine T ..... 1 gm.
Distilled water ..... 100 ml.
Warm gently 50-60°C.
Filter with Whatman's filter paper No. 1 (Solution stable for 10 days in refrigerator.)

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain with MAYER'S HEMATOXYLIN (s) for 30 seconds.
3. Rinse excess stain from the slides in running water and blue in tepid water for 5 minutes. (The hematoxylin is used to quench nuclear fluorescence.)
4. Place slides in Thioflavine T Solution for 3-8 minutes.
5. Rinse in water.
6. Differentiate in 1% ACETIC ACID (s) for 5 minutes.
7. Rinse in 2 changes of distilled water for 1 minute each.
8. Air dry.
9. Dip in xylene and mount.

Results:
For rat, mouse, rabbit, cat, dog, armadillo, pig and human. Guinea pig shows no results.
Juxtaglomerular granules - bright golden yellow
Background - pale green
Hyaline droplets and RBC - usually autofluorescent - flat greenish yellow or dull orange.
Fluorescence is best visualized with a BG-12 exciter filter in combination with an OG-1 barrier filter.

Reference: Janigan, D.T., Arch. Path. 79, pp. 370-375, 1965.
(Modification of Vassar and Culling Stain for Amyloid)

KERATIN AND MUCIN - Kreyberg

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) for 5 minutes.
3. Wash in running water for 5 minutes.
4. Stain in 1% PHLOXINE B (s) for 3 minutes.
5. Rinse in distilled water.
6. Differentiate quickly in 80% alcohol.
7. Rinse in tap water and distilled water.
8. Stain in ALCIAN BLUE (s) for 5 minutes.
9. Rinse in distilled water.
10. Stain in ORANGE G SOLUTION (KREYBERG) (s) for 13 minutes.
11. Dehydrate in alcohols, clear in xylene and mount.

Results:
Acid mucopolysaccharides - turquoise blue
Prekeratin and Keratin - orange to red-orange
Nuclei -orange to brown

Reference: Kreyberg, L., British J. of Cancer 25, 206-210, 1961.

LEUCINE - AMINOPEPTIDASE - Modified

Fixation:

Fresh frozen, cryostat sections, mounted.

Technique:

Cut cryostat sections at 6 microns. Use control of fresh frozen rat or guinea pig kidney. Prepare 6 slides. Do not reuse incubating solution.

Prepare:

Stock Substrate

L-leucyl-4-methoxy-B-napthylamide	8 mg.
Acetone	0.5 ml.
Distilled water	0.5 ml.

Incubating Medium

Fast Blue B Salt	10 mg.
ACETATE BUFFER (0.1 M, pH 6.5) (s)	10 ml.
Distilled water	9 ml.

Incubating Solution

Add Incubating Medium to Stock Substrate.

Staining Procedure:

1. Incubate slides and controls for 15 minutes to 2 hours at 37°C. Check at several time intervals.
2. Rinse in SALINE (s) for 2 minutes.
3. Immerse in 0.1 M CUPRIC SULFATE (s) for 2 minutes.
4. Rinse in SALINE (s).
5. Dehydrate in alcohols, clear in xylene and mount.

Results:

Sites of aminopeptidase activity will be bright red surrounded by yellow background.

Reference: Pearse, A.C., *Histochemistry Theoretical and Applied*, p. 913, 1961. 2nd. Ed. (modified at NIH by Dr. G. Glenner and B. Coolidge)

LEUKOCYTES - Sudan Black B

Fixation:
Air dried smears placed in Formalin vapor for 10 minutes.

Technique:
Smears of blood or bone marrow

Prepare:

Sudan Black B Stock Solution

Sudan Black B	0.3 gm.
Absolute alcohol	100 ml.

Buffer Solution

Dissolve:

Sodium Phosphate, Dibasic, 12-Hydrate ($Na_2HPO412H_2O$)	0.3 gm.
Distilled water	100 ml.

Add:

Phenol (dissolved in)	16 gm.
Absolute alcohol	30 ml.

Working Sudan Black B Solution

Buffer solution	40 ml.
Sudan Black B Stock Solution	60 ml.

Filter by suction. Stain satisfactory for 2-3 months.

Staining Procedure:

1. Rinse slides in running water.
2. Stain in Working Sudan Black Solution for 1 hour.
3. Wash slides with 70% alcohol for 2-3 minutes.
4. Wash in running water for 2-3 minutes.
5. Blot dry and counterstain with May-Grunwald-Giemsa.

Results:
Normal blood and bone marrow, granulocytes - faint localized cytoplasmic sudanophilia in the least mature cells and increased rapidly to strongly localized or heavy overall positively as maturation progresses.
Monocytic series - In a proportion of these cells, discrete sudanophilic granules are scattered over cytoplasm and nucleus alike. Auer bodies are stained.
Erythroid and lymphocytic cells - negative

Reference: Wintrobe, *Clinical Hematology,* p. 451, 6th Ed., 1969.

LIPOFUSCIN - Ferric Ferricyanide Reduction Technique

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 5-10 microns.

Prepare:
Ferric Ferricyanide Reagent
1% Potassium Ferricyanide ..... 4 ml.
1% Ferric Chloride ..... 30 ml.
Distilled water..... 6 ml.
Basic Fuchsin Solution
BASIC FUCHSIN, SATURATED AQUEOUS (s).... a few drops
1% ACETIC ACID (s) ..... 40 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Immerse slides in Ferric Ferricyanide Reagent for 10 minutes.
3. Wash in 1% ACETIC ACID (s)
4. Counterstain in Basic Fuchsin Solution for 5 minutes or until tissue is pink.
5. Rinse in 1% ACETIC ACID (s).
6. Dehydrate in alcohols, clear in xylene and mount.

Results:
Sites of reducing substances - blue
Background - pale pink or faint green
Nuclei - pink to red

Reference: Lillie, R.D., *Histological Technic and Practical Histochemistry,* p. 176, 2nd, Ed., 1954.

MAST CELLS (Guinea Pig) - Low Iron Diamine - Spicer

Fixation:
Carnoy's

Technique:
Cut paraffin sections at 6 microns

Prepare:
Low Iron Diamine Solution
Weigh 30 mg. of N, N-dimethyl-m-phenylenediamine (HC1)2 and 5 mg. of N, N-dimethyl-p-phenylenediamine (HC1). Add these reagents simultaneously to 50 ml. of distilled water. When dissolved, pour solution immediately into a coplin jar containing 0.5 ml. of 10% commercial (N.F.) FERRIC CHLORIDE SOLUTION (s).

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Oxidize in 1% PERIODIC ACID (s) for 10 minutes.
3. Wash in running water for 5 minutes.
4. Stain slides in the Low Iron Diamine solution for 18-24 hours.
5. Rinse quickly in distilled water.
6. Stain in ALCIAN BLUE SOLUTION (s) for 30 minutes - 1 hour.
7. Rinse in distilled water.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:
Guinea pig mast cells - dark blue to black
Background - light blue

Reference: Spicer, S.S., Outline of Mucopolysaccharide Histochemistry, Notes from Staff Conference, AFIP, Washington, D.C., October 31, 1960.

MAST CELLS - Toluidine Blue

Fixation:
Any

Technique:
Cut paraffin sections at 6 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Rinse slides in 60% alcohol.
3. Stain in TOLUIDINE BLUE (s) for 1 to 2 minutes.
4. Rinse quickly in tap water.
5. Dehydrate in acetone, 2 changes, 2 minutes each.
6. Clear in xylene and mount.

Results:
Mast cells - deep reddish purple
Background - faint blue

Comments: In addition to Toluidine Blue O, some other metachromatic dyes are: thionin, methylene blue, azures, crystal violet, cresyl violet, methyl violet, safranin O, celestine blue, gallocyanin and pinacyanole. The difficulty in preserving metachromasia lies in the dehydration of the tissues after staining. Increasing strengths of ethyl alcohol revert the dye back to the orthochromic form. Some workers use acetone, isopropyl or tertiary butyl alcohol for dehydration, followed by a clearing agent. Always stain at room temperature; heat can destroy metachromasia.

Reference: Humason, G.L., *Animal Tissue Techniques,* 3rd. Ed., W.H. Freeman & Co., pp. 346, 350, 1972.

MELANIN - Lillie

Fixation:

10% Formalin - avoid chromates.

Technique:

Cut paraffin sections at 6 microns. Use control slides.

Prepare:

2.5% Ferrous Sulfate (Must be fresh)

Potassium Ferricyanide-Acetic Solution

2% Potassium Ferricyanide ..... 20 ml.
2% Acetic acid ..... 20 ml.
(Must be mixed just before use)

Staining Proceudre:

1. Deparaffinize slides and hydrate to water.
2. Place slides in freshly prepared 2.5% Ferrous Sulfate for 1 hour.
3. Wash in distilled water for 20 minutes.
4. Place in freshly prepared Potassium Ferricyanide-Acetic Solution for 30 minutes.
5. Rinse in 1% ACETIC ACID (s).
6. Rinse in distilled water.
7. Counterstain in VAN GIESON SOLUTION (s) for 3 minutes.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:

Melanin - dark green
Collagen - red
Muscle and Cytoplasm -yellow

Reference: Lillie, R.D., *Histopathologic Technic and Practical Histochemistry*, p. 427, 3rd. Edition, 1965.

MELANIN - Schmorl

Fixation:
Formalin

Technique:
Frozen or paraffin sections cut at 6 microns.

Prepare:
Ferricyanide Solution
1% Ferric Chloride or Ferric Sulfate ..... 30 ml.
1% Potassium Ferricyanide (fresh) ..... 10 ml.
Use within 30 minutes.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Immerse slides in Ferricyanide Solution for 5 minutes.
3. Washing in running water for 1 minute.
4. Rinse quickly in distilled water.
5. Counterstain in 1% NEUTRAL RED (s) for 3 minutes.
6. Dehydrate rapidly in alcohols.
7. Clear in xylene and mount.

Results:
Melanin - dark blue
Argentaffin granules - dark blue
Lipofuscin - dark blue
Background - pink

Reference: Pearse, *Histochemistry, Theoretical and Applied*, p. 925, 2nd Ed., 1961.

MITOCHONDRIA - Regaud's

Fixation:
Regaud's Fluid

Technique:
Cut paraffin sections at 3 microns.

Prepare:
5% Iron Alum (Ferric Ammonium Sulfate)
2.5% Iron Alum

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Mordant in 5% Iron Alum for 2 hours.
3. Wash briefly in distilled water.
4. Stain in REGAUD'S HEMATOXYLIN (s) for 2 hours.
5. Wash in water.
6. Decolorize in 5% Iron Alum for a short time and place in 2.5% Iron Alum for detailed differentiation, controlling the result with microscope.
7. Wash in running water for 15-30 minutes.
8. Dehydrate in alcohols, clear in xylene and mount.

Results:
Mitochondria - black

Reference: Mallory, *Pathological Technique,* page 110, 1968.

MUCICARMINE - Mayer's (modified)

Fixation:
Any well-fixed tissue.

Technique:
Cut paraffin sections at 6 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in MAYER'S HEMATOXYLIN (s) for 1 minute.
3. Wash in running water for 5- 10 minutes.
4. Stain in MUCICARMINE (s) for 30 minutes.
5. Rinse in distilled water.
6. Rinse quickly in 95% alcohol.
7. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Mucin - deep rose to red
Cryptococcus - red
Nuclei - blue

Reference: Mallory, F.B., *Pathological Technique,* p. 130, 1968.

MUCOPOLYSACCHARIDES - Alcian Blue - PAS

Fixation:

10% Formalin or Carnoy's (Zenker's and Bouin's fixative tend to suppress staining).

Technique:

Cut paraffin sections at 6 microns. Acid mucopolysaccharide control - skin, umbilical cord. Neutral mucopolysaccharide control - chitin, fungi, pig, gastric mucosa.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in ALCIAN BLUE SOLUTION (s) for 30 minutes. (pH 2.5)
3. Wash in running water for 2 minutes.
4. Rinse in distilled water.
5. Place in 0.5% PERIODIC ACID (s) for 10 minutes.
6. Rinse in distilled water.
7. Place in SCHIFF'S REAGENT (s) for 15 minutes.
8. Rinse in 2 changes of SULFUROUS ACID RINSE (s) for 3 minutes each.
9. Wash in running water for 5 minutes.
10. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid mucopolysaccharides -blue
Neutral mucopolysaccharides - magenta
Cartilage, ground substance and majority of epithelial mucins - shades of purple to deep blue
Cell bodies of fungus - red to purple
Mucoid capsules of cryptococcus - blue

Reference: McManus and Mowry, *Staining Methods Histologic and Histochemical*, pp. 63-64, 1960.

MUCOPOLYSACCHARIDES - Astra Blue - Pioch

Fixation:

10% Formalin or Carnoy's. (Zenker's and Bouin's tend to suppress Alcian Blue and Astra Blue staining.)

Technique:

Cut paraffin sections at 6 microns

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in 1% ASTRA BLUE (s) for 5-10 minutes.
3. Rinse in 1% ACETIC WATER (s).
4. Rinse in distilled water.
5. Slides may be counterstained with NUCLEAR FAST RED (s) for 5 minutes or PAS procedure steps 2-6.
6. Dehydrate in alcohols, clear in xylene and mount.

Results:

Acid mucopolysaccharides - blue

Reference: Pioch, W., Virchows Arch. pp. 330: 337, 1957.

MUCOPOLYSACCHARIDES - Rinehart-Abul-Haj - Modified - Mowry

Fixation:

10% Formalin - avoid dichromate fixatives.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Sulfurous Rinse

1 M SODIUM BISULFITE (s)	10 ml.
Tap water	200 ml.

Potassium Ferrocyanide - HCl Solution

2% Potassium Ferrocyanide	1 part
1% HYDROCHLORIC ACID (s)	2 parts

Make and mix immediately before use.

Acidified Colloidal Iron

COLLOIDAL IRON, MULLER'S (s)	4 parts
Acetic acid	1 part

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Feulgen reaction
   a. Place slides in preheated (60°C.) N/HC_1 (s) for 10 minutes.
   b. Stain in SCHIFF'S REAGENT (s) for 15 minutes.
   c. Place in 3 changes of Sulfurous Rinse for 2 minutes each.
   d. Wash in running water 5-10 minutes.
   e. Wash in 80% alcohol.
3. Rinse in 3% ACETIC WATER (s).
4. Stain in Acidified Colloidal Iron for 10 minutes.
5. Wash slides in 5 or 6 changes of distilled water until sections are pale, almost colorless.
6. Immerse in Potassium Ferrocyanide - HCL Solution for 10 minutes.
7. Rinse well in two changes of distilled water.
8. Stain in VAN GIESON (s) for 7 minutes.
9. Wash quickly in 2 changes of 95% alcohol.
10. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:

Nuclei - red-purple
Cytoplasm, muscle and erythrocytes - yellow
Collagen and bone matrix - red
Mucins - blue, except that of the gastric surface epithelium
Cartilage matrix -variably blue to colorless

Reference: Rinehart, J.F. and Abul-Haj, S.K., Arch Path., 52, 189, 1951.

MYELIN, NERVE CELLS AND FIBERS - Luxol Fast Blue

Fixation:
10% Formalin

Technique:
Paraffin sections cut at 15-20 microns.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain overnight in LUXOL FAST BLUE SOLUTION (s) at 57°C.
3. Wash in 95% alcohol to remove excess stain.
4. Wash in distilled water - 2 changes.
5. Begin differentiation by quick immersion in 0.05% LITHIUM CARBONATE (s).
6. Continue differentiation in 70% alcohol until gray and white matter can be distinguished.
7. Wash in distilled water.
8. Finish differentiation by rinsing briefly in 0.05% LITHIUM CARBONATE (s) and through several changes of 70% alcohol until the greening blue of the white matter contrasts sharply with the colorless gray matter.
9. Wash thoroughly in distilled water.
10. Stain for 2 minutes in CRESYL VIOLET ACETATE (s) at 57°C. (preheated).
11. Differentiate in several changes of 95% alcohol.
12. Dehydrate in absolute alcohol, clear in xylene and mount.

Results:
Myelin - greenish blue
Cells - pink to violet

Reference: Kluver and Berrera: J. Neuropath. Exper. Neurol., 12: 400-403, 1953.

NERVE FIBERS - Holmes' Silver

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 8 microns. Use acid cleaned glassware.

Prepare:

Impregnating Solution

0.2 M BORIC ACID BUFFER SOLUTION (s)	55 ml.
0.05 M SODIUM BORATE BUFFER SOLUTION (s)	45 ml.
Mix in 500 ml. graduate and add 394 ml. distilled water (to 494 ml.)	
1% SILVER NITRATE (s)	1 ml.
10% Pyridine (pure)	5 ml.

Reducing Solution

Hydroquinone	1 gm.
Sodium Sulfite	5 gm.
Distilled water	100 ml.

2% Oxalic Acid

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in 20% SILVER NITRATE (s) in the dark at room temperature for 1-2 hours.
3. Wash in 3 changes of distilled water for 10 minutes.
4. Place in Impregnating Solution in a covered vessel at 37°C. for 24 hours.
5. Reduce in Hydroquinone Solution for not less than 2 minutes.
6. Wash in running water for 3 minutes.
7. Rinse well in distilled water.
8. Tone in 0.2% GOLD CHLORIDE (s) for 3 minutes.
9. Rinse well in distilled water.
10. Place in 2% Oxalic Acid until axons are black, 3-10 minutes.
11. Rinse well in distilled water.
12. Fix in 5% SODIUM THIOSULFATE (s) for 5 minutes.
13. Wash in running water 2-5 minutes.
14. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nerve fibers - black. This technique is excellent for guinea pig tissue.

Reference: Holmes, W., Ana. Record, 86: 157-187, 1943.

NISSL - Gallocyanin

Fixation:
10% Formalin, Zenker's or Zenker Formalin

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Gallocyanin Solution

Gallocyanin	0.15 gm.
Chromium Potassium Sulfate	5 gm.
Distilled water	100 ml.

Dissolve the chrome alum in warm water, add gallocyanin, and boil gently for 10 minutes. Cool and filter. The solution is stable for about one week.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain in Gallocyanin Solution at 56° for 1 hour or overnight at room temperature.
3. Rinse in water.
4. Dehydrate in alcohols, clear in xylene and mount.

Results:
Nissl substance -blue

Reference: Einarson, L.: Amer. J. Path. 8: 295-309, 1932.

PANCREAS - Aldehyde Fuchsin - Gomori Modification

Fixation:
Any well-fixed tissue

Technique:
Paraffin sections cut at 6 microns

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Place in LUGOL'S IODINE SOLUTION (s) for 20 minutes.
3. Rinse in distilled water.
4. Place in 5% HYPO (s) for 2 minutes.
5. Wash in tap water for 2 minutes.
6. Rinse in distilled water.
7. Rinse in 95% alcohol.
8. Stain in ALDEHYDE FUCHSIN (s) for 30 minutes. Should not be over 1 week old.
9. Rinse in 3 changes of 95% alcohol.
10. Rinse in distilled water.
11. Stain sections in CELESTINE BLUE (s) for 1-1 1/2 minutes, (if fresh, 1 minute.)
12. Rinse in distilled water.
13. Rinse in 95% alcohol (2 changes).
14. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Beta cells in Islets of Langerhans - rose
Nuclei - blue
Elastica - deep rose to purple

Reference: Gomori, G.L.: Am. J. Clin. Path. 20: 665-666, 1950.

PANCREAS - Beta Cells - Sieracki

Fixation:

10% Formalin or Bouin's

Technique:

Cut paraffin sections at 4-5 microns.

Prepare:

Mordant

Chromic Chloride (reagent grade) ..... 5 gm.
5% Acetic acid ..... 100 ml.
(Solution stable for 6 months)

Staining Solution

New Fuchsin..... 1 gm.
70% Alcohol..... 100 ml.
In a separate container, mix -
Hydrochloric acid ..... 2 ml.
Paraldehyde (USP) ..... 2 ml.
Add Paraldehyde-Acid mixture to the solution of New Fuchsin. Allow the stain to stand at room temperature for 24 hours or until it is a deep purple color. (Stable for 6 weeks in refrigerator).

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in Mordant for 72 hours.
3. Rinse well in water.
4. Rinse in 70% alcohol.
5. Stain for 6 hours in the Aldehyde-New Fuchsin Staining Solution.
6. Rinse in 70% alcohol.
7. Wash in running water 5-10 minutes.
8. Rinse in water.
9. Counterstain if desired with working solution of LIGHT GREEN (s) or FAST GREEN (s).
10. Rinse in 95% alcohol for 30 seconds.
11. Dehydrate in 100% alcohol for 30 seconds.
12. Clear in xylene and mount.

Results:

The granules of beta cells stain deep blue to purple in the pancreatic islet of man, dog, mouse, frog, guinea pig, rabbit and rat. As opposed to the argentaffin granules in Carcinoid tumors of the intestine, the beta granules in islet tumors are not readily demonstrable. This technique stains both non-neoplastic islets and beta cells in islet tumors.

Reference: Sieracki, J.C., et al: Stain Technology 35: pp. 67-69, 1960.

PANCREAS - Islet Cells
Chromium Hematoxylin-Phloxine-Gomori Modified

Fixation:
Bouin's preferred. 10% Formalin or Zenker-formol. Alpha cells - water soluble granules; beta cells - alcohol soluble granules.

Technique:
Cut paraffin sections at 2-3 microns.

Prepare:

Potassium Dichromate-Sulfuric Acid Solution

Potassium Dichromate	0.15 gm.
Distilled water	100 ml.
Sulfuric acid, concentrated	0.15 ml.

Hematoxylin Solution

Hematoxylin	0.5 gm.
Distilled water	50 ml.
When dissolved add -	
3% Potassium Chromium Sulfate (chrome alum)	50 ml.
Mix well and add:	
POTASSIUM DICHROMATE, 5% (s)	2 ml.
N/2 Sulfuric Acid (2.5%)	2 ml.

Allow solution to ripen for 48 hours. Can be used as long as metallic luster forms on surface after standing for 1 day. (4-8 weeks). Filter before use. Store in refrigerator.

Phloxine Solution

Phloxine B	0.5 gm.
Distilled water	100 ml.

5% Sodium Bisulfite

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Refix in Bouin's for 12-24 hours.
3. Wash in running water for 5 minutes.
4. Place in Potassium Dichromate-Sulfuric Acid Solution for 5 minutes.
5. Decolorize in 5% Sodium Bisulfite for 3-5 minutes.
6. Wash in running water for 5 minutes.
7. Stain in Hematoxylin Solution for 10-15 minutes or until beta cells are deep blue. Check under microscope.
8. Differentiate in 1% HYDROCHLORIC ACID (s) about 1 minute.
9. Wash in running water until section is clear blue - 5 minutes.
10. Stain with Phloxine Solution for 5 minutes.
11. Rinse briefly.
12. Treat with 5% PHOSPHOTUNGSTIC ACID (s) for 1 minute.
13. Wash in running water until sections turn red again - 5 minutes.
14. Differentiate in 95% alcohol. If sections are too red and the alpha cells do not stand out clearly, rinse for 15-20 seconds in 80% alcohol.
15. Dehydrate in alcohols, clear in xylene and mount.

Results:

Beta cells - blue
Alpha cells - red
Delta cells (not present in all animals) - pink to red and indistinguishable from alpha cells.

Reference: Gomori, G., Am. J. Path., 17, 397-399, 1941.

PANCREAS - Islet Cells - Glenner and Lillie

Fixation:

10% Formalin. Avoid acetic acid fixatives.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Eosin B - Aniline Blue Solution (Pancreas)

EOSIN B, 1% (s)	8.0 ml.
ANILINE BLUE, 1% (s)	2.0 ml.
CITRIC ACID M/10 (s)	1.4 ml.
SODIUM PHOSPHATE M/5 (s)	0.6 ml.
Distilled water	28.0 ml.

Adjust pH to 3.6.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain slides in pre-heated Eosin B-Aniline Blue Solution at 60°C. for 3 minutes. Cool to room temperature.
3. Wash in running water for 5 minutes.
4. Dehydrate in 50%, 80% and 100% acetone.
5. Clear in acetone-xylene and xylene and mount.

Results:

Enterochromaffin cells - blue-black
Goblet cells and mucins -light blue
Paneth cells - blue-black
Alpha cells of Pancreatic islet - red
Beta cells of Pancreatic islet - purple

Reference: Glenner, G.G. and Lillie, R.D., Stain Technology, 32: 187-190, 1957.

PANETH CELLS - Azure Eosin - Nocht

Fixation:

10% Formalin or Zenker's. Avoid acetic acid. It destroys zymogen and Paneth cell granules.

Technique:

Cut paraffin sections at 6 microns.

Prepare:

Buffered Azure Eosin Solution pH 4.1

0.1% Azure A	4 ml.
0.1% Eosin B	4 ml.
M/10 CITRIC ACID (s)	1.2 ml.
M/5 SODIUM PHOSPHATE (s)	0.8 ml.
Acetone (C.P.)	5 ml.
Distilled water	25 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in Buffered Azure Eosin Solution for 1 hour.
3. Dehydrate in 3 changes of acetone.
4. Rinse in equal parts of acetone and xylene.
5. Clear in 3 changes of xylene and mount.

Results:

Nuclei, tigroid, bacteria, rickettsiae - blue
Mast cells and basophils - blue
Calcium deposits - dark blue
Secretion granules in pancreatic and salivary gland acini and Paneth cells - pink
Cytoplasm of chief cells - blue
Inclusion bodies - pink
Cytoplasm of gastric gland parietal cells - pink

Note: The buffer mixture may be altered, lower pH levels giving redder effects, higher levels more blue. Demonstration of Paneth cells in rodents is more successful after animals have fasted for 8-18 hours.

Reference: Lillie, R.D., *Histopathologic Technic and Practical Histochemistry*, pp. 118-120, 1954. 2nd Ed.

PANETH CELLS - Napthalene Black

Fixation:

Any, except fixatives containing acetic acid. Demonstration of Paneth Cells in rodents is more successful after animals have fasted for 8-18 hours.

Technique:

Cut paraffin sections at 5-6 microns.

Prepare:

Napthalene Black Solution

Dissolve -
Napthalene Black (Amido Black 10B) ..... 0.5 gm.
in -
Distilled water ..... 50 ml.
and -
Propylene glycol ..... 50 ml.

Periodic Acid - Hotchkiss

Periodic acid ..... 0.8 gm.
Alcohol, absolute ..... 70 ml.
M/5 SODIUM ACETATE (s) ..... 10 ml.
Distilled water ..... 20 ml.

Reducing Bath - Hotchkiss

Potassium Iodide ..... 2 gm.
Sodium Thiosulfate ..... 2 gm.
Alcohol, absolute ..... 60 ml.
Distilled water ..... 40 ml.
20% Hydrochloric acid ..... 0.5 ml.
Store at 17° - 22° C. Stable for 14 days.

0.01% Lithium Carbonate

Staining Procedure:

1. Bring slides to 70% alcohol.
2. Treat in Periodic Acid Solution for 3-5 minutes.
3. Rinse in 70% alcohol.
4. Place in Reducing Bath for 1/2 to 1 minute.
5. Rinse in 70% alcohol.
6. Wash briefly in running water and rinse in distilled water.
7. Treat with SCHIFF'S REAGENT (s) for 5 minutes.
8. Wash in running water for 10 minutes.
9. Stain in Napthalene Black Solution for 15 minutes.
10. Wash in running water for 2 minutes.
11. Differentiate with 0.01% Lithium Carbonate until Paneth cell granules are well defined.
12. Wash in running water.
13. Dehydrate in alcohols, clear in xylene and mount.

Results:

Paneth cell granules and eosinophilic leukocyte granules - deep blue
Mucin - magenta
RBC greenish blue

Reference Bower and Chadwin, J. Clin. Path., 21, 107, 1968.

PAPANICOLAOU (Smears)

Fixation:

Ether-Alcohol for 15 minutes. Equal parts 95% alcohol and ether.

Technique:

Smears

Staining Procedure:

Remove smears from fixative and proceed as follows:

1. 80% alcohol - 10 dips.
2. 70% alcohol - 10 dips.
3. 50% alcohol - 10 dips.
4. Distilled water - 10 dips.
5. HARRIS HEMATOXYLIN (s) 1 1/4 to 1 1/2 minutes. Filter each day before use. Store in dark bottle when finished.
6. Distilled water - 10 dips.
7. 0.5% HCL - 7 dips.
8. Running tap water - 5 minutes.
9. Distilled water - 10 dips.
10. 50% alcohol - 10 dips.
11. 70% alcohol - 10 dips.
12. 80% alcohol -10 dips.
13. 95% alcohol - 10 dips.
14. ORANGE G6 (s) - 3/4 minutes.
15. 95% alcohol - 10 dips.
16. 95% alcohol - 10 dips.
17. EOSIN AZURE 50 (s) - 1 1/4 minutes.
18. 95% alcohol - 10 dips.
19. 95% alcohol - 10 dips.
20. 95% alcohol - 10 dips.
21. Absolute alcohol -10 dips.
22. Clear in xylene and mount.

Results:

Nuclei - blue with sharp details

Cytoplasm - shades of pink, yellow, green and gray.

Reference: Papanicolaou, G.N., *Atlas of Exfoliative Cytology,* 1954. (slightly modified at NIH)

PAS - TOLUIDINE BLUE - AURANTIA

Fixation:

10% Formalin or Zenker-Formol. Avoid acetic acid fixatives.*

Technique:

Cut paraffin sections at 5-6 microns.

Prepare:

Celestine Blue Solution

Celestine Blue B ..... 1 gm.
Sulfuric acid..... 0.5 ml.

Rub the dye to a paste with the acid until it becomes a friable mass. Reduce to coarse granules with a glass rod. Flood the granules with the following mixture:

Ferric alum ..... 2.5 gm.
Glycerin..... 14 ml.
Distilled water ..... 100 ml.

The ferric alum must be heated to 50°C. and stirred constantly as it is added to the Celestine blue granules.

Cool to room temperature and adjust the pH to 0.8 with concentrated sulfuric acid.

0.5% Toluidine Blue Solution

Aurantia Solution

Aurantia ..... 0.25 gm.
50% Alcohol..... 100 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in 1% PERIODIC ACID (s) for 2 minutes.
3. Rinse in distilled water.
4. Place in SCHIFF REAGENT (s) for 8 minutes.
5. Wash in running water for 10 minutes.
6. Stain with Celestine Blue Solution for 2 minutes.
7. Rinse in water.
8. Stain with MAYER'S HEMATOXYLIN (s) for 1 1/2 minutes.
9. Wash in running water for 5-10 minutes.
10. Stain with 0.5% Toluidine Blue Solution for 1 minute.
11. Rinse in water.
12. Differentiate in 70% alcohol until zymogen granules are deep blue and the background is clear. (This takes up to 1 minutes.) If difficult to differentiate - use 1% ACETIC ALCOHOL (s).
13. Stop differentiation by washing in running water.
14. Place slides in 0.25% Aurantia for 10 seconds.
15. Wash in running water.
16. Dehydrate quickly in tertiary butyl alcohol, clear in xylene and mount.

Results:

Nuclei - blue black
Epithelial lining cells and mucous neck cells, (mucin producing cells) - red
Zymogenic granules - blue
Parietal cells (HCl producing cells) and RBC - yellow
Background - shades of yellow-brown

Reference: Cook, H.C., Stain Tech., pp. 317-319, Sept. 1962.

*Paneth cell and zymogen granules of the salivary glands, pancreas and peptic glands are destroyed by acetic acid.

PEROXIDASE - Myeloperoxidase

Fixation:

Fix in 10% Alcoholic Formalin for 60 seconds. Rinse gently in water for 15-20 seconds.

Technique:

Smears of blood or bone marrow or organ imprints. Anti coagulants are not inhibitory. Fresh frozen sections may also be used.

Staining Procedure:

1. Place wet slides in MYELOPEROXIDASE pH 6 $\pm$ 0.5 INCUBATION MIXTURE (s) for 30 seconds.
2. Wash briefly in running water, dry and examine. If greater nuclear detail is desired, the stained preparations may be recounterstained in 1% cresyl violet acetate for 1 minute or in Giemsa for 10 minutes. Dry and mount.

Results:

Peroxidase activity - discrete blue granules in the cytoplasm of granulocytes and monocytes. Eosinophils are stained most intensely and are often tinged with brown-black or green-black. The cytoplasm of neutorphils is filled with blue dye. Monocytes exhibit weak to moderate activity. All other formed elements of blood and bone marrow, including, basophils are unstained.

Note: Benzidine dihydrochloride is a carcinogen and should be handled with care. It is less dangerous than benzidine (used in older techniques) because it is more granular.

Reference: Kaplow, L.S., Blood, 26, No. 2, 215-218, Aug. 1965.

PIGMENT REMOVAL

Melanin

1. Deparaffinize slides with xylene and wash in three changes of absolute alcohol. Coat the slides with celloidin (page ___.
2. Place the slides in .25% POTASSIUM PERMANGANATE (s) for one to several hours.
3. Decolorize sections in 5% OXALIC ACID (s) for 5 minutes.
4. Check under microscope. If melanin is not completely bleached, steps 2 and 3 may be repeated.
5. Wash in running water and rinse in distilled water.
6. Stain with desired stain.

Formalin and Malaria Pigments

1. Deparaffinize slides and hydrate to water.
2. Place in PICRIC ACID-SATURATED ALCOHOLIC(s) for at least 3 hours.
3. Wash well in running water.
4. Check under microscope. If pigment is not removed, replace in Picric Acid Solution overnight.
5. Wash well and stain with desired stain.

Mercuric Chloride Crystals

see "Routine Staining" - H & E

PINEAL BODY - Quay-Gomori

Fixation:

10% Formalin or Bouin's

Technique:

Cut paraffin sections at 7 microns. Mordant in Bouin's for 24 hours if Formalin fixed.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in POTASSIUM PERMANGANATE SOLUTION (s) for 1 minute.
3. Place in 3% SODIUM BISULFITE (s) until bleached.
4. Wash well in distilled water.
5. Stain in GOMORI'S CHROME HEMATOXYLIN (s) for 10-15 minutes.
6. Blue in tap water for approximately 1 minute.
7. Stain in 0.5% PHLOXINE (s) for 5 minutes.
8. Rinse in 5% PHOSPHOTUNGSTIC ACID (s) for 1 minute.
9. Wash in distilled water.
10. Stain in ORANGE G-ANILINE BLUE SOLUTION (s) for 1 minute.
11. Wash quickly in distilled water and 95% alchol.
12. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:

RBC -red
Pineal chromophils - dark orange or yellow
Pineal chromophobes
  Nuclei - blue
  Nucleous - orange to red

Reference: Quay, Experimental Cell Research, 10, pp. 541-568, 1956.

PITUITARY - Aldehyde Fuchsin - Gomori

Fixation:
Bouin's or mordant for 2 hours at 60°C. in Bouin's. Avoid Chromates.

Technique:
Paraffin sections cut at 3 or 4 microns.
Note: Allow 5 hours to complete.

Prepare:
0.1% Acetic Alcohol
95% Alcohol ..... 90 ml.
1% ACETIC ALCOHOL (s) ..... 10 ml.

Staining Procedure:
1. Deparaffinize slides and bring to 95% alcohol.
2. Place in LUGOL'S SOLUTION (s) for 20 minutes.
3. Rinse in distilled water.
4. Place in 5% HYPO (s) for 2 minutes, then wash in tap water for 2 minutes.
5. Rinse in distilled water.
6. Rinse in 95% alcohol.
7. Stain in ALDEHYDE FUCHSIN (s) for 30-45 minutes. Stain should not be over 1 week old.
8. Rinse in 3 changes of 95% alcohol.
9. Rinse in 0.1% Acetic Alcohol.
10. Place in AZOCARMINE G (s) (preheated at 58-60°C.) for 2 hours at 58-60°C.
11. Remove slides from oven and cool for 30 minutes.
12. Rinse in distilled water.
13. Differentiate in ANILINE ALCOHOL (s). Examine under microscope.
14. Stop differentiation by placing in 1% ACETIC ALCOHOL (s) for 2 minutes.
15. Rinse well in distilled water.
16. Place in 5% PHOSPHOTUNGSTIC ACID (s) for 2 hours. (Do not wash in water - blot semi-dry.
17. Place in Working Solution of FAST GREEN (s) for 30 minutes.
18. Rinse in 95% alcohol (two dips).
19. Dehydrate in absolute alcohols, clear in xylene and mount.

Results:
Pituitary beta cells - violet to purple
Elastic tissue and fibers - purple or deep blue
Mucin - purplish - deep blue
Nuclei - red
Connective tissue - green

Reference: Gomori, G: A.J. Clin. Path., Vol. 20, No. 7, July 1950.

PITUITARY - Glenner-Lillie

Fixation:
10% Formalin

Technique:
Cut paraffin sections at 6 microns.

Prepare:
Eosin B - Aniline Blue Solution

EOSIN B, 1% (s)	8.0 ml.
ANILINE BLUE, 1% (s)	2.0 ml.
CITRIC ACID, M/10 (s)	1.1 ml.
SODIUM PHOSPHATE, M/5 (s)	0.9 ml.
Distilled water	28.0 ml.

Staining Procedure:
1. Deparaffinize slides and hydrate to water.
2. Stain slides in Eosin B - Aniline Blue Solution in 60°C. oven for 1 hour. Let cool to room temperature. Place slides in cold water to set stain.
3. Wash in running water for 5 minutes.
4. Dehydrate quickly in 50%, 80% and 100% acetone.
5. Clear in acetone-xylene and xylene and mount.

Results:
Beta cell granules - blue to black
Acidophil cells - dark red
Chromophobe granules - slate gray to pale pink
Colloid - red to blue-violet
RBC - orange

Reference: Glenner, G.G. and Lillie, R.D., Stain Technology 32: 187-190, 1957.

PITUITARY - PAS-Orange G

Fixation:
10% Formalin or Zenker's

Technique:
Cut paraffin sections at 4 microns.

Staining Procedure:
1. Do PAS through Step 6.
2. Rinse in distilled water.
3. Stain with ORANGE G SOLUTION (s) for a few seconds.
4. Rinse quickly in distilled water.
5. Dehydrate in alcohols, clear in xylene and mount.

Results:
Pituitary alpha cells - red to purple granulation
Beta cells - orange-yellow

Reference: Pearse, *Histochemistry Theoretical and Applied*, p. 831, 2nd Ed., 1961.

RETICULUM - Gomori

Fixation:

Any well fixed tissue

Technique:

Cut paraffin sections at 6 microns. Use acid cleaned glassware for silver solutions. Sections do not loosen easily with this technique. Celloidin coating may be used.

Prepare:

2% Ferric Ammonium Sulfate Solution

10% Potassium Hydroxide Solution

Potassium Hydroxide	0.5 gm.
Distilled water	5 ml.

Ammoniacal Silver Solution - Make fresh

10% SILVER NITRATE SOLUTION (s)	20 ml.
10% Potassium Hydroxide Solution	4-5 ml.

Add:

28% Ammonia water, drop by drop, shaking continuously until the resulting precipitate is dissolved. Carefully add more 10% SILVER NITRATE SOLUTION (s) until the precipitate that forms disappears when solution is shaken. Make the solution up to twice its volume with distilled water. It can be kept in an acid clean stoppered bottle for two days.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Oxidize in 0.5% POTASSIUM PERMANGANATE (s) for 1 minute.
3. Wash in running water for 2 minutes.
4. Decolorize in 2% POTASSIUM METABISULFITE (s) for 1 minute.
5. Wash in running water for 2 minutes.
6. Place in 2% Ferric Ammonium Sulfate for 1 minute.
7. Wash in running water for 2 mintues.
8. Rinse in two changes of distilled water for 30 seconds each.
9. Place slides in Ammoniacal Silver Solution for 1 minute.
10. Rinse in distilled water for 20 seconds.
11. Reduce in 20% FORMALIN (s) for 3 minutes.
12. Wash in running water for 3 minutes.
13. Tone in 0.2% GOLD CHLORIDE (s) for 10 minutes until brown tones have disappeared.
14. Rinse in distilled water.
15. Place slides in 2% POTASSIUM METABISULFITE (s) for 1 minute.
16. Fix in 2% SODIUM THIOSULFATE (s) for 1 minute.
17. Wash in running water for 2 minutes.
18. Rinse in distilled water.

19. Counterstain lightly with MAYER'S HEMATOXYLIN (s) for 20-30 seconds. (NUCLEAR FAST RED (s) may be used instead of hematoxylin.)
20. Blue in running water.
21. Dehydrate in alcohols, clear in xylene and mount.

Results:

Fine reticulum fibers - black.

Reference: Mallory, F.B., *Pathological Technique,* pg. 164, 1968.

RETICULUM - Wilder's

Fixation:

10% Formalin, Zenker's or Helly's

Technique:

Cut paraffin sections at 6 microns. If sections tend to fall off slide, celloidin may be used. Use acid-cleaned glassware for steps 6-12.

Prepare:

Ammoniacal Silver Solution

To 5 ml. of 10% SILVER NITRATE SOLUTION (s) add 28% ammonia water drop by drop until precipitate which forms is dissolved. Add 5 ml. of 3.1% SODIUM HYDROXIDE (s) and just dissolve the resulting precipitate with a few drops of ammonia water. Make the solution up to 50 ml. with distilled water.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in 0.25% POTASSIUM PERMANGANATE (s) for 5 minutes.
3. Rinse in distilled water.
4. Place sections in HYDROBROMIC ACID SOLUTION (s) for 1 minute.
5. Wash well in distilled water.
6. Dip in 1% URANIUM NITRATE (s) for 5 seconds.
7. Wash well in distilled water.
8. Place slides in the Ammoniacal Silver Solution for 1 minute. Agitate.
9. Rinse well in 95% alcohol.
10. Place in WILDER'S REDUCING SOLUTION (s) for 1 minute.
11. Wash well in distilled water.
12. Tone in 0.5% GOLD CHLORIDE (s) for 1 minute or longer. Check under microscope until brown tones have disappeared.
13. Rinse well in distilled water.
14. Place in 5% SODIUM THIOSULFATE (s) for 2 minutes.
15. Wash in tap water 2-3 minutes.
16. Wash well in distilled water.
17. Counterstain lightly with MAYER'S HEMATOXYLIN (s) for 20-30 seconds. NUCLEAR FAST RED (s) may be used instead of hematoxylin.
18. Blue in running water.
19. Rinse in distilled water.
20. Dehydrate in alcohols, clear in xylene and mount.

Results:

Fine reticulum fibers - black
Collagen - rose

Reference: Wilder, H.C.: Amer. J. Path., 11:817-821, 1935.

RNA and DNA - Acridine Orange - Fluorescent

Fixation:

Any well fixed tissue.

Technique:

Use gelatinized slides. Paraffin sections cut at 6 microns or fresh frozen sections (fixed prior to staining). *DO NOT WORK UNDER FLUORESCENT LIGHT OR BRIGHT SUN LIGHT.*

Prepare:

Acridine Orange Working Solution

ACRIDINE ORANGE 0.1% (s) ..... 10 ml.
PHOSPHATE BUFFER pH 6 (s) ..... 90 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Rinse briefly in 1% ACETIC ACID (s).
3. Wash thoroughly in distilled water.
4. Stain in Acridine Orange Working Solution for 3 minutes.
5. Place in PHOSPHATE BUFFER pH 6 (s) for 1 minute.
6. Place in CALCIUM CHLORIDE 0.1 M SOLUTION (s) for 1 minute.
7. Wash thoroughly in two changes of PHOSPHATE BUFFER pH 6 (s).
8. Air dry.
9. Dip in xylene and cover with a low fluorescence mounting media.

Stained slides will fade slowly, but if not exposed to excessive UV light, are acceptable for two months or more.

Results:

DNA of chromatin of nucleus - green
Connective tissue fibrils and cornified epithelia -green
Mucus - dull green
Leukocytes - bright green
RNA of basophilic structures of cytoplasm and main portion of nucleoli - red and orange fluorescences.
Sites of strongly acidic groups - deep carmine.
Malignant cells under low power - brilliant to flaming red-orange.
Proliferating malignant cells under high power - intense fluorescence.

Cytoplasm - flaming red-orange containing reddish granules, patches or fine granules.
Nuclei - yellowish hues, yellow-orange to yellow-green.
Nucleoli - brilliant orange-red.

Degenerating and necrotic malignant cells with loss of cytoplasmic RNA - faint orange or brick red. (RNA often concentrated at periphery in a rim of dark brick red).

Nuclei - brilliant green, green-yellow or pale yellow-gray.
Nucleoli, often enlarged and multiple - orange-red.

Reference: Von Bertalanffy, Ludwig, and Bickis; J. Histochem. and Cytochem. 4, 481-493, 1956.

RNA - Methyl Green-Pyronin pH 5.3

Fixation:

Carnoy's or Telly's. Zenker Acetic gives moderate results. Do not fix in Bouin's.

Technique:

Cut paraffin sections at 4-5 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in MGP SOLUTION pH 5.3 (s) for 45 seconds to 3 minutes, depending upon the age of the stain.
3. Differentiate in 2 changes of distilled water.
4. Differentiate and dehydrate in 2 changes of acetone (quick dips).
5. Change to equal parts of acetone-xylene (agitate rapidly).
6. Clear in xylene and mount.

Note: Always stain a few extra slides to find the proper technique for differentiation. Better results can sometime be obtained by using tap water, 95% alcohol and absolute alcohol. If the blue is too intense, tap water will remove it more quickly than distilled water. If the red is too intense, 95% alcohol will remove it effectively. If both methyl green and pyronin stain too intensively, a weak solution of acetic acid in 80% alcohol differentiates well.

The cytoplasm and connective tissue should be pastel pink in contrast to the deep red of the RNA.

Results:

Cytoplasmic and nucleolar ribonucleic acid - red
Nuclei - blue
Mast cells - orange

To prove that the red color is due to RNA, another sample can be digested for 3 hours at 60°C. with a 0.1% solution of commercial ribonuclease buffered with sodium barbital to a pH of 6.75 before staining with the MGP solution.

Reference: This is a modification of Kurnick, found in Pearse, *Histochemistry*, p. 825, 2nd Edition, 1961.

RNA - Methyl Green-Pyronin - Rogers

Fixation:

Any well-fixed tissue except Bouin's. This method works well on formalin-fixed tissues.

Technique:

Cut paraffin sections at 3-4 microns.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Stain in MGP SOLUTION-ROGERS (s) for 5-10 minutes.
3. Rinse quickly in 80% alcohol.
4. Rinse quickly in distilled water.
5. Blot with bibulous paper and dehydrate in tertiary butyl alcohols.
6. Check under microscope. If stained too red, repeat steps 3 and 4. If nuclei are blue-green continue directly to xylene and mount.

Results:

DNA - green to bluish-green
Nucleoli and cytoplasmic RNA appears pink to red.

Reference: E. Rogers, G. d'Ablaing, R. Lukes, L.A. County USC Medical Center, L.A. Calif. (Paper presented at AFIP seminar).

SPIROCHETES - Warthin Starry - AFIP Modification

Fixation:

10% Formalin. Avoid chromate.

Technique:

Cut paraffin sections at 6 microns. Use acid cleaned glassware. Use control slide. Do not use metal forceps.

Prepare:

Acidulated Water

Triple distilled water ..... 1000 ml.
Adjust pH to 4.0 with 1% citric acid. (It is sometimes desirable to use pH 3.6 to prolong development.)

1% Silver Nitrate Impregnation Solution

Silver Nitrate ..... 1 gm.
Acidulated water ..... 100 ml.

2% Silver Nitrate Developing Solution

Silver Nitrate ..... 2 gm.
Acidulated water ..... 100 ml.

5% Gelatin Solution

Gelatin ..... 10 gm.
Acidulated water ..... 200 ml.

0.15% Hydroquinone Solution

Hydroquinone ..... 0.15 gm.
Acidulated water ..... 100 ml.
Place 2% Silver Nitrate, 5% Gelatin and Hydroquinone Solutions in a water bath at 54°C.

Developer Solution

Combine in order given and mix well after each addition. Prepare immediately before use.
2% Silver Nitrate Developing Solution ..... 10.5 ml.
5% Gelatin Solution ..... 26.25 ml.
Hydroquinone Solution ..... 14 ml.

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place slides in preheated 1% Silver Nitrate Impregnating Solution in 43%C. water bath or incubator for 30 minutes.
3. Prepare developer solution.
4. Place slides in Developer Solution and allow to develop until sections are light brown or yellow. Check control under microscope. Spirochetes are black and background tan to yellow.
5. Wash slides well but quickly in hot tap water, approximately 56°C. (If overdevelopment has occurred, sections can be treated with iodine and hypo for color removal and restained.)
6. Rinse in distilled water.
7. Dehydrate in alcohols, clear in xylene and mount.

Results:

Spirochetes and Donovan bodies - black
Background - pale yellow to light brown

Reference: Warthin, A.S. and Starry, A.C: Amer. Jour. Syph., 4: 97, January, 1920.

TRYPTOPHAN

Fixation:

10% Formalin or Glutaraldehyde

Technique:

Cut paraffin sections at 6 microns

Prepare:

Condensing Solution

Dissolve:

p-dimethylaminobenzaldehyde ..... 1 gm.
Acetic acid ..... 30 ml.
Add:
Hydrochloric acid ..... 10 ml.

Oxidizing Solution

Acetic acid ..... 40 ml.
1 N HYDROCHLORIC ACID (s) ..... 0.3 ml.
1% SODIUM NITRITE ..... 0.7 ml.
(Make just prior to step 7)

Staining Procedure:

1. Deparaffinize slides and hydrate to water.
2. Place in PICRIC ACID-SATURATED AQUEOUS (s) for 2 hours. This step is necessary for a permanent preparation.
3. Take slides through graded alcohols, rinsing in absolute alcohol three times. Slides must be thoroughly dehydrated.
4. Rinse well in concentrated Acetic Acid.
5. Place in Condensing Solution for 20 minutes.
6. Wash gently in Acetic Acid three times (30 seconds, 1 minute, and 1 minute.)
7. Place in freshly prepared Oxidizing Solution for 5 minutes.
8. Wash gently in three changes of Acetic Acid for 1 minute each.
9. Clear in 4:1, 1:1, 1:4 Acetic Acid-xylene and through two changes of xylene and mount.

Results:

Sites of protein-bound tryptophan stain various intensities of blue.

Reference: Glenner, J. Histochem. and Cytochem., 5, 297, 1957.
Adams: J. Clin, Path., 10, 65, 1957.

URIC ACID - Gomori Burtner-Lillie Modification

Fixation:

Absolute alcohol or Carnoy's

Technique:

Paraffin sections cut at 6 microns. Start tissue processing in absolute alcohol. Float sections onto slides with 95% alcohol. Dry sections at 37°C. for 2 hours. (Some urates are water soluble).

Prepare:

Holmes-Methenamine Solution pH 9

METHENAMINE SILVER (s)	35 ml.
M/5 BORIC ACID SOLUTION (s)	1.6 ml.
M/20 SODIUM BORATE (s)	6.4 ml.

Staining Procedure:

1. Deparaffinize slides and wash in absolute alcohol.
2. Transfer slides directly to 37°C. preheated Holmes Methenamine Solution and allow to remain until uric acid deposits are black - 15-60 minutes.
3. Place in 5% SODIUM THIOSULFATE (s) for 3 minutes.
4. Wash in running water for 3 minutes.
5. Counterstain in EOSIN (s) for 1 minute.
6. Dehydrate in alcohols, clear in xylene and mount.

Results:

Urates - black
Other tissue elements - pink

Reference: Pearse, *Histochemistry, Theoretical and Applied,* p. 949, 2nd. Ed., 1961.

WRIGHT'S STAIN

Fixation:

Methyl alcohol for 1 minute. Air dry.

Technique:

Blood or bone marrow smears. A well-made slide or coverslip preparation is extremely important and this skill should be perfected.

Staining Procedure:

1. Place slide or coverslip on staining rack and cover with freshly filtered WRIGHT'S STAIN (s) for 1 1/2 minutes.
2. Add an equal amount of distilled water to the stain and mix by gently blowing. The stain must not overflow the edges of the slides. Allow to stain an additional 3 minutes. There should be a green metallic scum on the surface of the stain.
3. Flush slides with distilled water while they are still flat to avoid precipitate from settling on the cells. Wipe back of slide and stand on end to drain.
4. Air dry, dip in xylene and mount.

Results:

Polymorphonuclear leukocytes - purplish-blue nuclei, pink cytoplasm with violet granules.
Lymphocytes - dark blue nuclei, clear blue cytoplasm.
Monocytes - blue nuclei, light blue webby cytoplasm.
Eosinophils - blue nuclei, large red granules over cytoplasm
Basophils - large black granules covering surface of cell
RBC -pink
Platelets - red-purple

Note:
Distilled water should be neutral or slightly acid. A phosphate buffer of 6.4 may be used.
If the stain is too alkaline the red cells will be blue or green. A drop of acetic acid may be added to the stain.
If the stain is too acid the nuclei of the leukocytes will be pale blue and red cells bright red or orange. A drop of ammonia water may be added to the stain.
Blowing the breath on the stain buffer mixture will also acidify the solution with carbon dioxide and make the slides pinker.
To test the reaction of the distilled water, place a few crystals of hematoxylin in a test tube and add 5 ml. of water.
If the water is neutral, it will become pale lavender-pink within 2 minutes.
If the water is acid, it becomes yellow and remains so longer than 5 minutes.
If the water is alkaline, it becomes reddish-purlie immediately, or before 1 minute.

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